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Development and Assessment of Procedures for Collection, Chemical Characterization and Mutagenicity Testing of Ambient Air

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June 1983

DEVELOPMENT AND ASSESSMENT OF PROCEDURES FOR COLLECTION, CHEMICAL CHARACTERIZATION AND MUTAGENICITY TESTING OF AMBIENT AIR

by

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16. ABSTRACT

The overall objective of this Research Triangle Institute pilot study was to develop and evaluate a minimal testing protocol which could detect and quantify the mutagenic potential of ambient air. The cost-effective biological and chemical testing protocol developed under this program outlines sampling, chemical fractionation/identification, and bioassay techniques which are applicable to air pollution testing of both ambient air particles and vapors. More specifically, this study: (1) evaluated the three stage Massive Air Volume Sampler (MAVS), developed by Battelle for collection of air particle samples; (2) developed and utilized a chemical fractionation scheme for solvent extraction of organic compounds from ambient air particles; (3) evaluated a GC/MS/Computer analysis system designed to chemically identify signature mutagens/carcinogens; and (4) utilized and evaluated modifications of the Ames/Salmonella typhimurium mutagenicity assay for ambient air particles and vapors.

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The complexities of environmental problems originate in the deep interdependent relationships between the various physical and biological segments of man's natural and social world. Solutions to these environmental problems require an integrated program of research and development using input from a number of disciplines. The Health Effects Research Laboratory, Research Triangle Park, NC and Cincinnati, OH conducts a coordinated environmental health research program in toxicology, epidemiology and clinical studies using human volunteer subjects. Wide ranges of pollutants known or suspected to cause health problems are studied. The research focuses on air pollutants, water pollutants, toxic substances, hazardous wastes, pesticides, and non-ionizing radiation. The laboratory participates in the development and revision of air and water quality criteria and health assessment documents on pollutants for which regulatory actions are being considered. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of environmental regulatory decisions involving the protection of the health and welfare of all U.S. inhabitants.

The overall objective of this Research Triangle Institute pilot study was to develop and evaluate a minimal testing protocol which could detect and quantify the mutagenic/carcinogenic potential of ambient air. The cost-effective biological and chemical testing protocol developed under this program outlines sampling, chemical fractionation/identification, and bioassay techniques which are applicable to air pollution testing of both ambient air particles and vapors. More specifically, this study: (1) evaluated the three-stage Massive Air Volume Sampler (MAVS), developed by Battelle® for collection of air particle samples; (2) developed and utilized a chemical fractionation scheme for solvent extraction of organic compounds from ambient air particles; (3) evaluated a GC/MS Computer analysis system designed to chemically identify signature mutagens/carcinogens; and (4) utilized and evaluated modifications of the Ames/*Salmonella typhimurium* mutagenicity assay for ambient air particles and vapors.

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Dr. Alan R. Kolber died as the result of an airplane accident on Sunday, June 7, 1981. The authors of this report mourn his tragic and untimely death. Although Dr. Kolber was not directly responsible for the authorship of this report, he was the project leader for this program at its completion. In addition, he did contribute to the development of the preincubation technique for detection of vapor-phase organic mutagens. His keen intellect and unique personality will be missed.

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ABSTRACT

The overall objective of this Research Triangle Institute (RTI) pilot study was to develop and evaluate a minimal testing protocol which could detect and quantify the mutagenic (and by implication carcinogenic) potential of ambient air. The cost-effective biological and chemical testing protocol developed under this program outlines sampling, chemical fractionation/identification, and bioassay techniques which are applicable to air pollution testing of ambient air particles and vapors. More specifically, this study: (1) evaluated the three stage Massive Air Volume Sampler (MAVS), developed by Battelle® for collection of air particle samples; (2) developed and utilized a chemical fractionation scheme for solvent extraction of organic compounds from ambient air particles; (3) evaluated a GC/MS/Computer analysis system designed to chemically identify signature mutagens/carcinogens; and (4) utilized and evaluated modifications of the Ames/Salmonella typhimurium mutagenicity assay for ambient air particles and vapors.

Studies concerned with the development of methodology utilized air particles that were collected with the MAVS in three sizes: <1.7, 1.7-3.5, and >3.5-20 μm (mean particle diameter). Although problems were encountered with the MAVS, limited amounts of ambient air particulate samples were collected at: South Charleston, West Virginia; Baton Rouge, Louisiana; Lake Charles, Louisiana; Beaumont, Texas; Houston, Texas; Upland, California; and Elizabeth, New Jersey. The collected particles were removed from the impactor and precipitator plates and were fractionated into general chemical-classes with a solvent scheme that was developed and verified for its chemical and biological efficiency. The scheme fractionated the crude extract into six general chemical-classes: organic acids, organic bases, nonpolar neutrals (NPN), polynuclear aromatic hydrocarbons (PNA), polar neutrals (PN), and cyclohexane insoluble (CHI) fractions. The mass of each chemical fraction was quantified, and the crude extract and the chemical-class fractions were then tested for mutagenicity in the Ames/Salmonella bioassay. Either the standard pour plate incorporation assay or a newly developed agar well diffusion assay was employed for testing. A bioassay priority scheme was developed to optimize the amount of information obtainable from a limited amount of sample.

Results suggested that the PNA, PN, acidic, and basic fractions of the crude extract were generally mutagenic in the Ames/Salmonella assay. Mutagenic activity was detected both with and without S9 metabolic activation, which indicated the presence of both indirect-acting and direct-acting mutagens, respectively. Qualitative chemical analysis with a GC/MS/Computer analysis system identified mutagenic/carcinogenic compounds in the mutagenic fractions. These studies demonstrated both

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the usefulness and necessity of chemical fractionation. The developed solvent fractionation scheme unmasked mutagenicity that was not detected in the crude organic extract. Potent mutagenic fractions were identified by concentration of quantitatively minor chemical-classes (such as the organic basic fraction) which allowed a more meaningful chemical and biological analysis to be performed. Unfortunately however, quantitative mutagenic analysis was inconclusive. The small amount of air particles collected and the amount of extractable organic material (10-15% w/w of the particles), which required further fractionation into chemical-classes, precluded sufficient dose responsive mutagenicity testing with all five Salmonella strains. Testing could not be consistently performed in the presence and absence of an S9 metabolic activation system, and replication of results was not possible. In view of these deficiencies, no absolute conclusions could be reached concerning the relative mutagenicity of the air particles collected from these sites. In spite of these difficulties, the mutagenicity results from this program did qualitatively identify probable mutagenic compounds and chemical-classes. However, these results were only preliminary and should not be utilized in health risk assessment. The purpose of this program was to develop and evaluate testing procedures; it was not designed to evaluate the mutagenicity of specific sites.

Mutagenicity testing of vapor-phase organic samples in the Ames/Salmonella assay from these sites was not conducted because an adequate quantitative method was not available. A preincubation technique was therefore developed to quantitatively measure vapor-phase organic mutagens in field samples collected on Tenax® resin. The modified preincubation technique was tested with pure mutagenic vapor-phase organic compounds (ethylene oxide, propylene oxide, butylene oxide, styrene oxide, ethylene dibromide, and vinylidene chloride). Results suggested increased sensitivity with the preincubation technique when comparison was made to results with the standard pour plate technique. Vapor-phase samples collected from the above cities on Tenax® cartridges contained signature mutagenic and carcinogenic compounds identified by GC/MS/Computer analysis. Mutagenic components in the vapor-phase organic samples consisted mainly of short-chain chlorinated hydrocarbons such as tetrachloroethylene. The combined Tenax®-resin/preincubation technique can now be utilized to identify and evaluate potentially mutagenic ambient air vapors.

In conclusion, a chemical fractionation scheme, a modified Ames/Salmonella plate assay (agar well diffusion test), and a biological priority testing scheme were developed and utilized for air particle testing. A preincubation technique was developed for detection of vapor-phase mutagens with the Ames/Salmonella assay, and was tested with known mutagenic vapor-phase compounds. GC/MS/Computer analysis was evaluated for chemical identification of mutagenic/carcinogenic components. Mutagenicity was qualitatively detected in certain chemical-class fractions from particulate samples from seven U. S. cities. Greater particulate sample amounts (≥ 50 g) are required for quantitative analysis. Signature mutagenic and/or carcinogenic signature compounds were identified in certain vapor-phase and particulate samples from

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these sites. Guidelines for future biological and chemical air pollution studies were developed. These initial developmental studies indicated the usefulness and necessity of an integrated biological-chemical approach for analysis of ambient air pollutants and the need for future quantitative studies at industrial and rural sites.

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LIST OF ABBREVIATIONS AND SYMBOLS

ABBREVIATIONS

BAP	-- Benzo(a)pyrene
NPN	-- nonpolar neutral fraction
PNA	-- polynuclear aromatic hydrocarbon fraction
PN	-- polar neutral fraction
CHI	-- cyclohexane insoluble fraction
POM	-- particulate organic matter, as solid or liquid particles
VPO	-- vapor-phase organics
S9	-- supernatant of liver obtained at 9,000 g
MAVS	-- Massive Air Volume Sampler
DMSO	-- Dimethylsulfoxide (spectral-grade)
+MA	-- S9 from Aroclor-induced rat liver
-MA	-- No S9 addition

SYMBOLS

<	-- less than
>	-- greater than
μm	-- microns (10^{-6} meters)
mg	-- milligrams (10^{-3} grams)
μg	-- micrograms (10^{-6} grams)

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SECTION 1

INTRODUCTION

This report summarizes the results of a three year study of ambient air pollution initiated in May 1977 and concluded in June 1980. This study was sponsored and funded by the Health Effects Research Laboratory (HERL) of the United States Environmental Protection Agency. The goal of this research was to develop and evaluate a protocol for initial evaluation of ambient air pollutants. The protocol developed under this program defines a minimal biological and chemical testing procedure required to initially measure and identify potential mutagenic compounds in ambient air. This developed testing protocol includes sampling, fractionation, chemical identification, and microbial mutagenicity methods for identification and quantification of toxic organic compounds in ambient air. Methods for both particulate organic matter (POM) and vapor-phase organic (VPO) samples were developed and evaluated.

An integrated biological-chemical-engineering approach was utilized for the assessment of environmental air pollutants. This multidisciplinary approach has also been successful in evaluating and identifying mutagens present in other complex environmental mixtures, such as coal gasification effluents, fly ash from coal-fired power plants, drinking water, shale oil, synthetic fuels, automobile and diesel exhaust, and cigarette smoke condensate (Ames, 1979; Waters et al., 1979). However, this interdisciplinary approach has only been possible since the development of the Salmonella mammalian microsome mutagenicity assay, developed by Dr. Bruce Ames and his colleagues in 1975 (Ames et al., 1975b). This bacterial mutagenicity test is rapid and economical, requires little space, and detects many pure mutagens (McCann et al., 1975; McCann and Ames, 1976) at nanogram to microgram levels. With addition of a mammalian liver extract (S9), the Ames assay can detect many mutagens which require metabolic activation (Ames et al., 1973). The assay is based on reversion of histidine-requiring mutants to histidine independence after interaction with mutagenic compounds. Since its development in 1975, the Ames assay has been utilized to detect a wide variety of environmental and pure mutagens (Ames et al., 1975a; Durston and Ames, 1974; Kier et al., 1974; McCann et al., 1975; McCann and Ames, 1976; Waters et al., 1979). The correlation of mutagenic with carcinogenic potential of many compounds has been demonstrated to exceed 80% (Commoner et al., 1976; McCann et al., 1976; Sugimura et al., 1976). This assay can therefore detect carcinogens as mutagens in the majority of cases (McCann and Ames, 1977).

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The utility of this assay for detection of pure and environmental mutagens has led to the wide acceptance of the *Salmonella* assay as the test-of-choice for initial mutagenicity assessment. This three year air pollution development program, which was initiated in 1977, was a pilot study to determine if this assay, which at that time was a "novel" system, could detect mutagenic compounds in ambient air in a cost-effective and timely manner. Since that time, numerous other investigators have utilized the Ames/*Salmonella* bacterial mutagenesis assay to detect mutagens in ambient air. For example, initial studies by Pitts et al. (1977) demonstrated mutagenic activity for organic extracts of air particles collected at eight urban California sites, but not for particles collected at a rural site. Although the mutagenicity of the particulate matter collected by Pitts et al. (1977) was not enhanced by S9 metabolic activation, the mutagenicity of another California sample collected by Flessel (1977) was increased by S9 addition. A quantitative study performed by Tokiwa et al. (1980) on air particulate collected from Japanese cities found higher mutagenicity in organic extracts from industrial areas when compared to those from rural areas. Both direct- and indirect-acting mutagens were detected in the Tokiwa study and similar findings have been reported since 1977 (Tokiwa et al., 1977; Tolcott and Wei, 1977; Dehnen et al., 1977; Commoner et al., 1978; Teranishi et al., 1978; Tolcott and Harger, 1979; Löfroth, 1979; Alfheim and Moller, 1979; Moller and Alfheim, 1980). A general overview of the collection, chemical characterization, and mutagenicity testing of organic compounds extracted from ambient air particles was recently published by Hughes et al. (1980).

In addition to the development of the Ames/*Salmonella* mutagenicity assay, comprehensive studies of ambient air particles were made possible by other recent technical advances. The adaptation of chemical fractionation techniques originally designed to separate cigarette smoke condensate into chemical-classes (Swain et al., 1969) has served as a model for the study of many other types of complex environmental organic mixtures (Commoner, 1979; Huisingh et al., 1979; Pelroy and Peterson, 1979; Guerin et al., 1979) such as air particulates, diesel exhaust, shale oil, and liquidified coal samples. Such techniques reduce the complexity of the organic mixture and thereby permit meaningful chemical analysis and biological testing to be conducted. For example, fractionation permitted meaningful chemical identification of potential mutagenic/carcinogenic signature compounds in general chemical-classes of ambient air particulate samples (Pellizzari et al., 1979). Each chemical-class fraction contained hundreds of compounds; without fractionation meaningful chemical results were not attainable (Pellizzari, 1979).

The sampling of particles from very large volumes of air has been made possible by the development of a Massive Air Volume Sampler (MAVS) (Henry and Mitchell, 1978), which can sample over 20,000 m^3 of air in 24 hr, and can fractionate particles by size. Size-fractionation of particles is important because the smaller sized particles (<2 μm) present a greater surface area per unit weight for adsorption of organic pollutants. The smaller particles are also more easily inhaled and deposited in the lung, and are more difficult to expel (Lippman et al., 1979; Schlesinger

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and Lippman, 1978; Yeh et al., 1976; Natusch and Wallace, 1974). Particle size is therefore an important factor when attempting to quantify the mutagenicity of ambient air particles; collection equipment should therefore be capable of collecting particles based on size.

The methods development phase of this EPA program utilized both pure compounds and ambient air samples to evaluate various techniques. Ambient air particles collected from seven cities were utilized to evaluate the Massive Air Volume Sampler, the chemical fractionation scheme, the GC/MS/Computer analysis system, and the Ames/*Salmonella* assay. Pure mutagenic compounds were utilized to verify the biological and chemical efficiency of the chemical fractionation scheme, and to evaluate the modifications to the Ames assay that were developed for both ambient air particulate and vapor-phase organic samples. A modification of the Ames plate incorporation technique, an agar well diffusion assay, was developed to optimize the information available from a limited sample size. The development of the agar well technique was necessary because only a small fraction of the total particulate sample was organic (10-20%). The agar well diffusion test was evaluated with known mutagenic compounds. Results suggested that the agar well assay could qualitatively detect both direct- and indirect-acting mutagens and toxicity on a single plate, which greatly increased the total amount of information obtainable from each chemical-class fraction. In addition, a chemical fractionation scheme was developed, which divided crude solvent extract from air particulate samples into six chemical-classes (organic acids and bases, polar and nonpolar neutrals, polynuclear aromatic hydrocarbons, and cyclohexane insoluble chemical-class fractions). The scheme was evaluated for both chemical and biological efficiency to determine if fractionation was quantitative, if known chemicals were properly fractionated (qualitative accuracy), and if fractionation influenced the mutagenic potential of known mutagenic compounds. The fractionation scheme allowed the identification (by GC/MS/Computer analysis) of mutagenic and carcinogenic compounds in mutagenically active fractions.

In addition to the development of methods for analysis of ambient air particles, a second major objective of this study was to develop and evaluate biological and chemical techniques which would quantify and identify organic vapor-phase mutagens present in ambient air. Volatile substances, which are vapors at ambient temperatures, have been shown to be a greater component of ambient air pollutants than are solid particulate matter. Duce (1978) cited data that measured the vapor-phase organic pollutant load at 50 x 10^{11} g/day, and the particulate organic matter load at 4 x 10^{11} g/day. Consequently, the levels of vapor-phase organics are generally 10-50 times greater than the levels of particulate organic matter (Duce, 1978). Mutagenicity testing has been conducted on pure vapor-phase mutagens/carcinogens that have been chemically identified in ambient air, but no research has been performed directly on ambient air vapors. The majority of the vapor research has been conducted with vinyl chloride and its metabolites (Bartsch, 1975; Loprieno et al., 1976; Bridges, 1978; Malaveille et al., 1975; Rannug et al., 1976). However, these vapor-phase compounds were difficult to evaluate quantitatively for mutagenic potential due to their volatility and aqueous-

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insolubility (Rosenkranz et al., 1979). Consequently, a sensitive quantitative technique to collect and measure the mutagens present as vapors in ambient air was developed under this program. Tenax® cartridges developed by Pellizzari (1979) were utilized for vapor collection and chemical analysis of field samples. A preincubation technique, modified after the technique originally developed by Yahagi and co-workers (Yahagi et al., 1975, 1977; Nagao et al., 1977) was developed and tested with pure vapor-phase mutagens. The preincubation technique was 3-10x more sensitive to vapor-phase mutagens identified in ambient air than the standard pour plate assay (vinylidene chloride; styrene, propylene, ethylene and butylene oxides; and ethylene dibromide). The development of the preincubation technique, in tandem with Tenax® collected field samples, allows future chemical and biological analysis of potentially mutagenic vapor-phase organic field samples.

This development program utilized actual field samples from seven cities to evaluate the techniques developed under this program. Both mutagenic and chemical testing was performed. The biological and chemical results suggested that potential mutagenic compounds were present in both air particle and vapor-phase samples. These results were preliminary and qualitative, and were not intended for use in risk assessment evaluation. Air particulate was sampled with the MAVS and subsequently fractioned into: organic acids and bases, polar and nonpolar neutrals, polynuclear aromatic hydrocarbons, and cyclohexane insoluble fractions. Fractions were then chemically quantified, signature mutagens were qualitatively identified by GC/MS/Computer analysis, and each fraction (when available) was tested for mutagenicity with the Ames/*Salmonella* assay. Mutagenic analysis of vapor-phase field samples was not performed because a quantitative biological technique was not available; however, mutagenic vapor-phase mutagens were chemically identified with GC/MS/Computer analysis in field samples.

This report summarizes the research conducted to develop and evaluate reliable cost-effective techniques for air pollution analysis. The format of the report details RTI's efforts for both ambient air particle and vapor-phase samples. Detailed experimental procedures are contained in the appendices for: Sampling and Chemical Analysis of VPOs (Appendix A); Protocol for the Ames/*Salmonella* Assay (Appendix B); and the Protocol for the Agar Well Diffusion Assay (Appendix C). The Abstracts and Publications that have resulted from this research are contained in Appendix D.

This pilot research program has answered many questions about ambient air pollution. However, significant research problems still remain including: chemical and biological analysis of the inorganics in ambient air; optimization and quantification of chemical extraction procedures; and verification and quantification of mutagenicity in particulate and vapor-phase organic field samples. It is hoped that the research described within this report will encourage and assist further efforts in this important research field.

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SECTION 2

CONCLUSIONS

* A minimal biological and chemical testing protocol was developed to evaluate the mutagenic potential of ambient air particles and vapors.

* A Massive Air Volume Sampler (MAVS), which was utilized in this study, size-fractionated ambient air particles into three sizes: <1.7 μm, 1.7-35 μm, and 3.5-20 μm. This size-fractionation was important because the smaller sized particles (i.e., <1.7 μm) contained the greatest amount of surface area per unit weight, were the most respirable particle size, contained the greatest measurable amount of signature carcinogens/mutagens per unit weight, and were the most mutagenic particle size overall.

* A chemical fractionation scheme was developed to solvent extract organic compounds from the particles. The scheme fractionated the total crude organic extract into six general chemical-classes: organic acids and bases, polar and nonpolar neutrals, polynuclear aromatic hydrocarbons, and cyclohexane insoluble fractions. Mixtures of pure mutagenic compounds were fractionated to verify the chemical and biological efficiency of this scheme. Chemical fractionation was necessary for chemical and biological analysis of ambient air. Advantages of chemical fractionation included: lessening of the toxicity present in the crude organic extract; concentration of minor, but highly mutagenic components; identification of mutagenic chemical-classes; and identification of signature mutagenic and carcinogenic compounds within these chemical-classes by GC/MS/Computer analysis.

* A modification of the standard Ames/Salmonella plate incorporation assay was developed to evaluate the mutagenic potential of chemical-classes from ambient air particles. This method, the agar well diffusion assay, allowed multiple parameters to be measured on a single plate (toxicity, mutagenicity, activation requirements, and dose range finding) which permitted the evaluation of mutagenic compounds present in air particles.

* Ambient air particles wre collected from seven U.S. geographical sites (South Charleston, West Virginia; Baton Rouge, Louisiana; Lake Charles, Louisiana; Beaumont, Texas; Houston, Texas; Upland, California; and Elizabeth, New Jersey). Particulate samples were utilized to evaluate the methods developed during this program. The particles were size classified, chemically fractionated, and tested for mutagenicity in the Ames/Salmonella mammalian microsome mutagenicity assay. Both the

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standard plate incorporation technique and the agar well difussion assay (developed under this program) were utilized. Signature mutagens were identified with a GC/MS/Computer system. Results suggested that: (1) the mutagenic activity was primarily present in the PNA, PN, acid, and base fractions; (2) the mutagenicity increased as the surface area/unit mass of particulate increased (*i.e.*, as the diameter of the particle decreased; (3) the chemical fractionation scheme unmasked mutagenicity which was not detected in the crude organic extract; (4) toxic effects present in the crude extract were reduced after fractionation; (5) PNA and PN fractions generally required metabolic activation while the acid and base fractions (the other two active fractions) generally did not require S9 addition--the NPN were generally not mutagenic; (6) both frameshift and base substitution mutagens were detected; (7) when sample size for chemical-class fractions was less than 10 mg, the agar well diffusion test allowed initial mutagenic analysis.

* A GC/MS/Computer analysis system identified signature mutagenic/carcinogenic compounds in the acid, base, PNA and PN classes from ambient air particles. The cyclohexane insoluble fractions were not tested due to either heavy contamination by fungi and bacteria or to insufficient sample size. Chemical idenitification of signature mutagenic compounds in mutagenic fractions strengthened the information obtained from field samples.

* Ambient air vapor samples were not tested for mutagenicity because an adequate quantitative method was not available. Consequently, a modification of the preincubation technique (in liquid suspension) was developed for utilization in field testing of ambient air vapors. This preincubation technique was developed and tested against the standard Ames/*Salmonella* assay with both stationary and logarithmically grown cells of *Salmonella* tester strain TA100. Six known vapor-phase mutagens were tested: butylene oxide, propylene oxide, ethylene oxide, ethylene dibromide, styrene oxide, and vinylidine chloride. Results from this testing suggested that: (1) the preincubation technique (with log-phase cells) was generally superior for detection of mutagenicity of six known vapor-phase mutagens, when comparison was made to the pour plate assay with either stationary- or log-phase cells; (2) the increased sensitivity of the preincubation assay was demonstrated by reduced variance at all dosage levels and by increased ability to detect vapor-phase mutagens, which was statistically verified by an increase in the initial mutagenicity slope (total revertants/μg) for six vapor-phase compounds tested; and (3) this Tenax®/preincubation technique is applicable to future ambient air vapor-phase field studies. The development of these procedures permits future quantitative chemical and biological analysis of potentially mutagenic ambient air vapors.

* Ambient air vapor-phase organic field samples were qualitatively analyzed with a GC/MS/Computer system. Sites tested included: Elizabeth, New Jersey; Lake Charles, Louisiana; Beaumont, Texas; and Houston, Texas. Signature mutagenic and carcinogenic compounds identified included: benzene and benzene isomers, chlorinated short-chain hydrocarbons (chloroform, carbon tetrachloride, trichloroethylene), and vinyl and vinylidene chloride.

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SECTION 3

RECOMMENDATIONS

The primary purpose of this program was to develop a testing protocol for future biological and chemical analysis of ambient air vapors and particles. Testing was performed on ambient air particles and vapors from various geographical locations as an aid in methods development; this qualitative mutagenic and chemical data was not intended for use in health risk assessment. However, these preliminary results suggest the need for further quantitative evaluations of similar cities. A rural site should be included as a negative control.

A minimal chemical and biological testing protocol to initially evaluate mutagenic and toxic potential of ambient air particles is outlined below.

* Size classification and chemical fractionation of organic extracts obtained from air particles should be performed. Initial biological and chemical testing requires a minimum of 30 g of particulate sample, since the percentage of organic material accounted for only 10-20% of the total weight of air particulate samples. This amount of total particulate sample (>30 g) would allow chemical and biological evaluation of the crude organic extract and the chemical-class fractions. Testing of both the crude extract and subsequent fractions will allow investigation into synergistic and antagonistic effects which may occur in the crude organic extract. An actual particulate sample should be fractionated and then reconstructed to evaluate the effect of fractionation on mutagenic potential. Adequate dose responsive mutagenicity testing requires a minimum of 50 mg of sample for each chemical-class fraction.

* Chemical identification of signature mutagenic/carcinogenic compounds in the mutagenically active fractions should be coupled with quantification of these compounds in relation to amount of air sampled. Additional analytical techniques, such as HPLC and LC/MS, should be employed to verify the results from the GC/MS/Computer analysis.

* Atmospheric conditions, time of year, sampling date and duration of sampling, and exact location of sampling site should be documented and reported, as all of these conditions can affect the toxic potential of ambient air.

* The mutagenic potential of the cyclohexane insoluble fraction should be investigated further.

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* Ames/Salmonella mutagenicity testing should be performed with the quantitative plate incorporation assay. Quantitative toxicity testing aids in the determination of nontoxic dose ranges for subsequent mutagenicity testing. Preferred doses for initial toxicity testing are: 1,000, 500, 100, 10, and 1 µg/plate. Samples should be tested both with and without S9 addition when only one tester strain can be utilized. Multiple doses (a minimum of five doses) should be tested, both with and without S9 metabolic activation. The standard S9 should be derived from rat liver which has been induced with Aroclor 1254. If mutagenic activity requiring S9 metabolic activation is initially detected, additional testing should be performed to determine optimal S9 concentrations. It is desirable to perform extensive testing with one Salmonella tester strain, rather than to perform minimal testing with all five tester strains, when sample size is less than 50 mg. Replicate dose response curves should be generated for active fractions. Strain priority for testing generally should be: TA98, TA100, TA1535, TA1537, and TA1538. The strain priority for testing can be different where specific information is required to fill in data gaps.

* The preincubation assay for vapor-phase mutagen detection requires further development to increase the sensitivity of the S9 metabolic activation system in liquid culture. The Tenax®/preincubation technique could then be utilized to evaluate the mutagenicity of vapor-phase organic field samples.

* Additional research to verify and standardize the chemical fractionation scheme should be conducted. Optimal solvents for the extraction of organics from ambient air particles need to be determined. Collection, fractionation, and bioassay systems should be optimized for sensitivity, accuracy, and reproducibility of results.

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SECTION 4

DEVELOPMENT OF METHODS

PARTICULATE ORGANIC MATTER (POM)

Methods for Collection of Ambient Air Particles

The collection of ambient air particulate samples for this study employed the Massive Air Volume Sampler (MAVS), adapted from the design of Henry and Mitchell (1978), as shown in Figure 1. The MAVS collected ambient air particles at 18 m^3/min and size-fractionated air particles into three separate size classes: <1.7 µm, 1.7-3.5 µm, and >3.5-20 µm fractions. The smallest fraction (<1.7 µm) was collected by a electrostatic precipitator; the other two classes (1.7-3.5 µm and >3.5-20 µm) of particles were impacted on Teflon® plates. The impactor plates were unitized and housed in a drawer of the impactor assembly. The electrostatic assembly was a separate unit. Upon collection of air particles, the plates were left in the assembly module; the module was removed, placed in a sealable aluminum hardshell shipping case, and transported to the laboratory. The entire impactor and electrostatic assemblies were placed in a specially constructed glove-box which was purged with helium or argon. Individual plates from each unit were removed and particles were scraped into a Pyrex® beaker which was tared as a single operation in the glove-box. Yellow lights were utilized for illumination to minimize photochemical reactions by the organics on the particles. Once recovery of the particles was completed for all plates in an assembly, the total particulate sample was transferred to glass vials with teflon-lined screw caps, purged with inert gas, sealed, wrapped in aluminum foil, and stored in a freezer until biological and chemical characterization could be initiated.

Development of a Chemical Fractionation Scheme for Particulate Organic Matter

The chemical fractionation scheme for solvent extraction of organic compounds from air particles was inspired by the work of Swain et al. (1969). The solvent partition scheme (Figure 2) generated six chemical groups: organic acids, organic bases, polynuclear aromatic hydrocarbons (PNA), polar neutrals (PN), nonpolar neutrals (NPN), and cyclohexane insolubles (CHI). An initial fractionation scheme (Figure 3) developed by Pellizzari et al. (1979) yielded 13 fractions, and was utilized on the two air particulate sample from South Charleston, West Virginia. Results indicated that this initial 13 chemical-class fractionation scheme resulted in ambiguously separated fractions and greatly reduced

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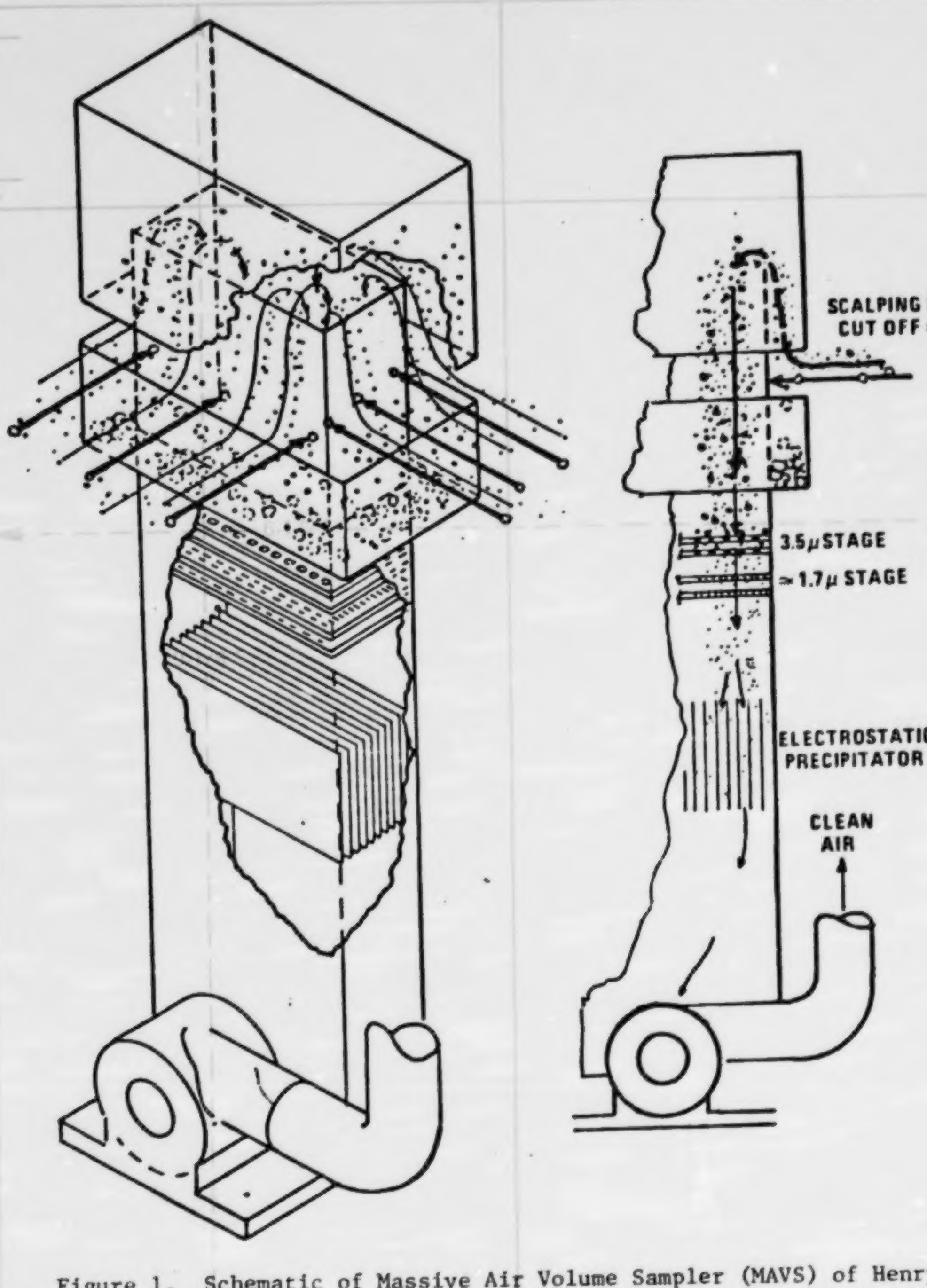
SCALPING STAGE, CUT OFF ≈ 20 μ
3.5 μ STAGE
≈ 1.7 μ STAGE
ELECTROSTATIC PRECIPITATOR
CLEAN AIR

Figure 1. Schematic of Massive Air Volume Sampler (MAVS) of Henry and Mitchell (1978), which was utilized to collect and size fractionate air particles for this study.

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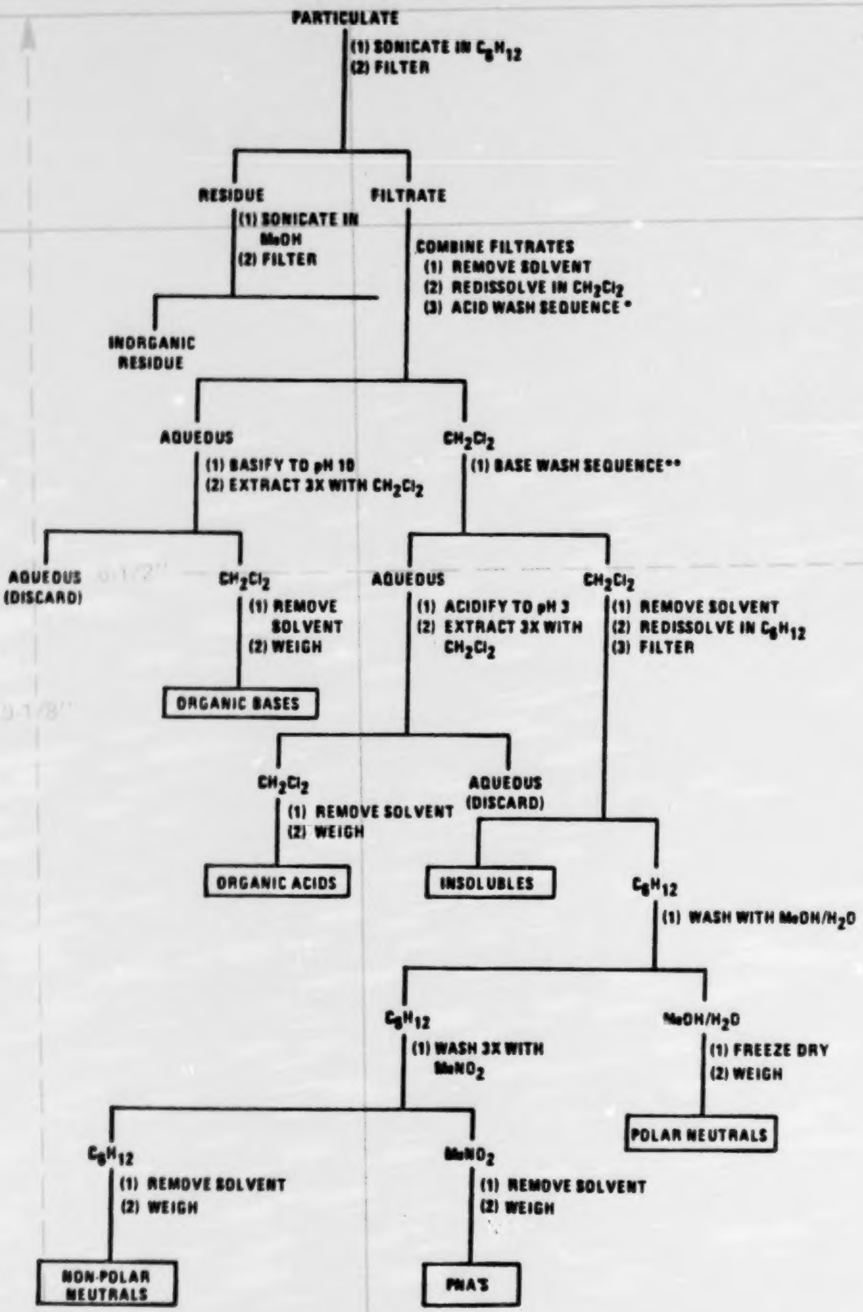
PARTICULATE
(1) SONICATE IN C_6H_{12}
(2) FILTER
RESIDUE
FILTRATE
(1) SONICATE IN MeOH
(2) FILTER
COMBINE FILTRATES
(1) REMOVE SOLVENT
(2) REDISSOLVE IN CH_2Cl_2
(3) ACID WASH SEQUENCE *
INORGANIC RESIDUE
AQUEOUS
CH_2Cl_2
(1) BASIFY TO pH 10
(2) EXTRACT 3X WITH CH_2Cl_2
(1) BASE WASH SEQUENCE**
AQUEOUS (DISCARD)
CH_2Cl_2
AQUEOUS
CH_2Cl_2
(1) REMOVE SOLVENT
(2) WEIGH
(1) ACIDIFY TO pH 3
(2) EXTRACT 3X WITH CH_2Cl_2
(1) REMOVE SOLVENT
(2) REDISSOLVE IN C_6H_{12}
(3) FILTER
ORGANIC BASES
CH_2Cl_2
AQUEOUS (DISCARD)
(1) REMOVE SOLVENT
(2) WEIGH
ORGANIC ACIDS
INSOLUBLES
C_6H_{12}
(1) WASH WITH $MeOH/H_2O$
C_6H_{12}
$MeOH/H_2O$
(1) WASH 3X WITH $MeNO_2$
(1) FREEZE DRY
(2) WEIGH
POLAR NEUTRALS
C_6H_{12}
$MeNO_2$
(1) REMOVE SOLVENT
(2) WEIGH
(1) REMOVE SOLVENT
(2) WEIGH
NON-POLAR NEUTRALS
PNA'S

*ACID WASH SEQUENCE 2X WITH 1N H_2SO_4, 1X WITH 2N H_2SO_4

**BASE WASH SEQUENCE 3X WITH 1 N NaOH

Figure 2. Final chemical-class fractionation scheme. Boxes indicate points for biological and chemical characterization.

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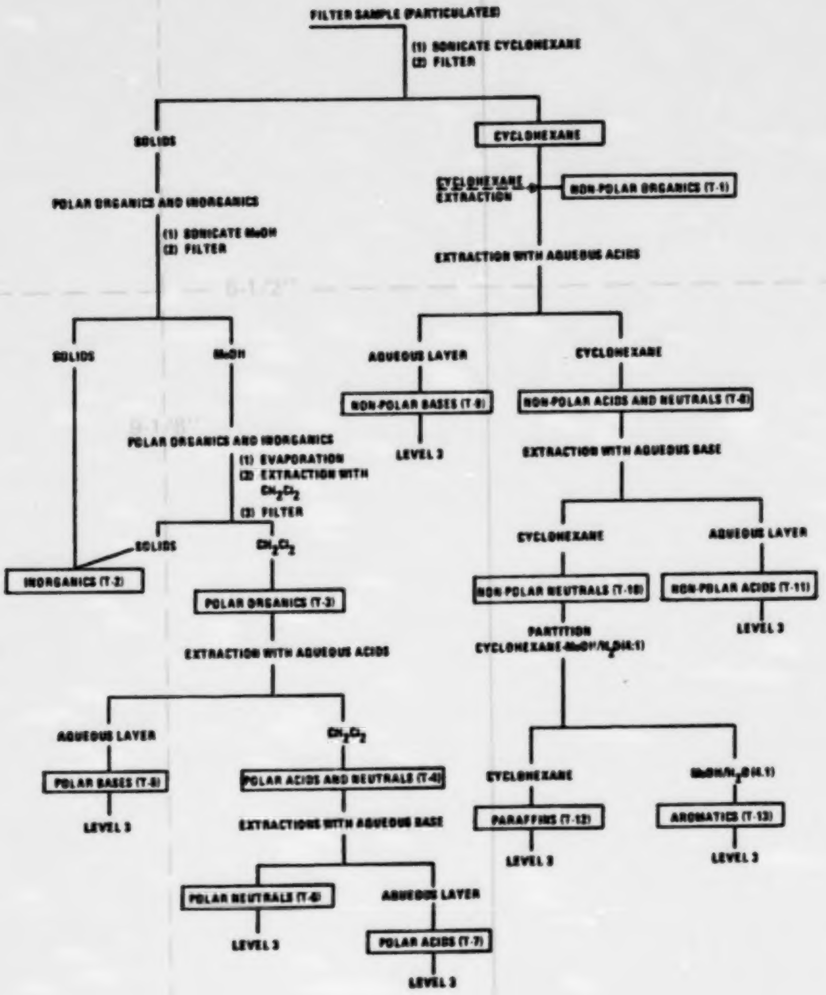
FILTER SAMPLE (PARTICULATES)
(1) SONICATE CYCLOHEXANE
(2) FILTER
SOLIDS
CYCLOHEXANE
POLAR ORGANICS AND INORGANICS
CYCLOHEXANE EXTRACTION
NON-POLAR ORGANICS (T-1)
(1) SONICATE MeOH
(2) FILTER
EXTRACTION WITH AQUEOUS ACIDS
SOLIDS
MeOH
AQUEOUS LAYER
CYCLOHEXANE
NON-POLAR BASES (T-9)
NON-POLAR ACIDS AND NEUTRALS (T-8)
LEVEL 3
POLAR ORGANICS AND INORGANICS
(1) EVAPORATION
(2) EXTRACTION WITH CH_2Cl_2
(3) FILTER
EXTRACTION WITH AQUEOUS BASE
SOLIDS
CH_2Cl_2
CYCLOHEXANE
AQUEOUS LAYER
INORGANICS (T-2)
POLAR ORGANICS (T-3)
NON-POLAR NEUTRALS (T-10)
NON-POLAR ACIDS (T-11)
LEVEL 3
PARTITION
CYCLOHEXANE-MeOH/H_2O(4:1)
EXTRACTION WITH AQUEOUS ACIDS
AQUEOUS LAYER
CH_2Cl_2
POLAR BASES (T-5)
POLAR ACIDS AND NEUTRALS (T-4)
CYCLOHEXANE
MeOH/H_2O(4:1)
LEVEL 3
EXTRACTIONS WITH AQUEOUS BASE
PARAFFINS (T-12)
AROMATICS (T-13)
LEVEL 3
LEVEL 3
POLAR NEUTRALS (T-6)
AQUEOUS LAYER
LEVEL 3
POLAR ACIDS (T-7)
LEVEL 3

Figure 3. Initial fractionation scheme. Boxes indicate ponts for biological and chemical characterization.

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the sample size per class. The initial scheme was therefore modified to yield six fractions, which reduced the number of fractions and increased sample size within each fraction. The initial scheme was based on work by Lee et al. (1976), and after significant modification (Pellizzari et al., 1979), led to the final scheme depicted in Figure 2, which was utilized on the six subsequent particle samples tested under this program.

The methodology for this six chemical-class fraction scheme (Figure 2) involved subjecting air particles (approximately 1.0 g) to an ultrasonic bath in 100 ml of cyclohexane (Burdick-Jackson) for 30 min. The sample was then filtered through a Teflon® filter (0.5 µm pore size). The filtrate was evaporated to dryness using a Rotovap®. The solids retained by the filter were taken up in 100 ml of methanol (B-J) and subjected to an ultrasonic bath for 30 min. The sample was then filtered through the Teflon® filter and the filtrate taken to dryness. The filtrates were combined, dried, weighed, and then redissolved in CH_2Cl_2. This solution was then extracted two times with an equal volume of 10% H_2SO_4, followed by an equal volume of 20% H_2SO_4. The aqueous phases were combined and washed with CH_2Cl_2, which was combined with the original CH_2Cl_2 phase. The aqueous phase was cooled in an ice bath, and then adjusted to pH 10 with 25% NaOH. It was then extracted three times with CH_2Cl_2 to generate the "organic bases". The aqueous phase was discarded. The original CH_2Cl_2 phase was extracted three times with 5% NaOH. The NaOH phases were combined and washed with CH_2Cl_2. The CH_2Cl_2 phase was then combined with the original CH_2Cl_2 phase. The combined NaOH phases were placed in an ice bath and acidified to pH 3.0 with 20% H_2SO_4. This phase was then extracted three times with CH_2Cl_2 to give "organic acids." The remaining aqueous phase was discarded.

The original CH_2Cl_2 phase was taken to dryness (Rotovap®), then reconstituted in cyclohexane and filtered through a Teflon® filter (0.5 µm). The cyclohexane filtrate was extracted three times with an equal volume of methanol:H_2O (4:1) solution. The methanol:H_2O extract was concentrated (Rotovap®), the H_2O was removed by freeze-drying to give "polar neutrals." The cyclohexane phase was extracted three times with an equal volume of nitromethane. The nitromethane phases were combined and evaporated to dryness to give the "PNAs." The cyclohexane phase was taken to dryness (Rotovap®) to give "nonpolar neutrals."

Evaluation of the Final Chemical-Class Fractionation Scheme

Assessment of the chemical and biological efficiency of the chemical-class fractionation scheme was evaluated in three ways. Initially, authentic commercially available chemicals representative of each chemical-class were mixed and fractionated by the scheme (Figure 2). A benzoic acid/phenol mixture represented the organic acid fraction; quinoline represented the organic base fraction; hexadecane represented the nonpolar neutral fraction; phenanthrene represented the PNA fraction; and ethylene glycol represented the polar neutral fraction. The results are presented in Table 1. Experiments were performed with both a large and small initial mass for each chemical-class. The percent recovery of these chemical compounds was greater than 70%; most classes had a greater

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TABLE 1. RECOVERY VERIFICATION OF KNOWN CLASS-CHEMICALS WITH THE FINAL CHEMICAL FRACTIONATION SCHEME (Figure 2)

Compound	Class	Amount added (mg)	Amount recovered (mg)	Percent recovered
Large initial mass				
Benzoic acid/phenol	Organic acid	41.2/39.0	74.0	92.3
Quinoline	Organic base	42.4	40.2	94.8
Hexadecane	Non-polar neutral	45.1	10.0	22.2[a]
Phenanthrene	PNA	45.4	34.4	75.8
Ethylene glycol	Polar neutral	44.6	43.4	97.3
Small initial mass				
Benzoic acid/phenol	Organic acid	0.8/6.4	7.0	97.2
Quinoline	Organic base	5.5	5.3	96.4
Hexadecane	Non-polar neutral	8.6	6.9	80.2
Phenanthrene	PNA	4.3	4.3	100.0
Ethylene glycol	Polar neutral	5.0	3.6	72.0

[a] Low value of recovery due to experimental error; recovery of hexadecane with a small initial mass (lower portion of table) demonstrated acceptable percentage recovery (80.2%).

than 90% recovery rate. The one exception to the above recovery rates was the NPN-hexadecane, which demonstrated an unusually low recovery rate of 22% with a large initial mass (45.1 mg). This low percent recovery was unusually low, compared to the recovery of hexadecane with a small initial mass (8.6 mg) which was greater than 80%. The low percent recovery for the large initial mass may be due to loss of volatilized sample. These results suggested that the fractionation scheme was capable of yielding quantitatively high recoveries of known chemical-class compounds from a complex mixture.

Secondly, known mutagenic compounds, representative of the five chemical-classes, were mixed and fractionated. This experiment was performed to measure the capability of the fractionation scheme to quantitatively recover known chemical-class mutagens. The chemicals chosen to represent each chemical-class were: 9-aminoacridine (organic base), 20-methylcholanthrene (PNA), 5-nitrofuroic acid (organic acid), phenol (polar neutral), and hexadecane (nonpolar neutrals). The phenol (PN) and hexadecane (NPN) were not known mutagens but were chosen as being representative of their particular chemical-class. The percent chemical recovery of these mutagens after fractionation is presented in Table 2. Recovery for all fractions was similar to the first experiment, since recoveries ranged from 62-105% for the PNA, acid, PN, and NPN fractions. The percent recovery in the basic fraction was 189.7%; this spillover more than accounted for the low recovery in the four other fractions. This experiment therefore suggested that chemical recovery of known chemical-class mutagens was reasonable, but that a spillover problem existed in the organic basic fraction.

The third evaluation was an assessment of the biological potential of mutagens before and after chemical fractionation. This experiment was performed to evaluate the effect of the organic solvents on a known mixture of mutagenic compounds. The chemicals chosen to represent each chemical-class were: 9- aminoacridine (organic base), 20-methylcholanthrene (PNA), 5-nitrofuroic acid (organic acid), phenol (polar neutral), and hexadecane (NPN). These compounds were tested before and after chemical fractionation with strains TA1535, TA1537, TA1538, TA98, and TA100. Results are presented in Table 3. Mutagenic activity for the PNA (20-methylcholanthrene) increased 2-fold after fractionation for TA98 with S9 metabolic activation at the 100 μg/plate dose, but was similar at the 50 μg/plate before and after fractionation. In addition, activity for this PNA was only detected in the PNA fraction, and required strain TA98 with S9 metabolic activation for detection. Activity which required TA98 and S9 metabolic activation was not detected in any other class. Mutagenic recovery of the direct-acting base (9-aminoacridine [9AA]) was reduced after fractionation, since only 26.2% of the original activity at 500 μg was recovered. Mutagenicity of the basic fraction (9AA) at 100 μg/plate was lost after fractionation. However, in spite of these spillovers between chemical-classes, mutagenic activity was qualitatively determined in the expected chemical-class, with the proper strain and under the proper metabolic activation requirements after fractionation for both the PNA and basic fractions. Mutagenic activity was not detected in any of the other chemical-classes with any strain,

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TABLE 2. CHEMICAL-CLASS MUTAGENS USED TO VERIFY THE CHEMICAL EFFICACY OF THE FINAL CHEMICAL FRACTIONATION SCHEME (Figure 2)

Chemical	Chemical class	Before fractionation (mg)	After fractionation (mg)	Percent Chemical Recovery
9-aminoacridine	base	22.3	42.3	189.7
20- methylcholanthrene	PNA	23.3	14.5	62.2
5-nitrofuroic acid	acid	22.5	15.3	68.0
phenol	polar neutral	21.7	20.2	93.1
hexadecane	non-polar neutrals	24.4	25.7	105.3
		114.2	118.0	103.6

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TABLE 3. MUTAGENIC EVALUATION OF KNOWN MUTAGENS BEFORE AND AFTER CHEMICAL FRACTIONATION

Compound	Strain	No. colonies before fractionation	Mutagenic ratio	No. colonies after fractionation	Mutagenic ratio[a]	Percent recovery of initial mutagenicity
9-aminoacridine (base)						
500 µg	TA1537	1,731	133[b]	452	34.8[b]	26.2
100 µg		670	52[b]	13	1	1.9
20-methylchlor-anthrene (MCA)[c]						
100 µg	TA98	186	6.2[b]	397	13.2[b]	213.6
50 µg		237	7.9[b]	258	8.5[b]	108.9
2-nitrofuroic acid (acid)						
500 µg	TA1535	0[d]	0	43	1.5	e
125 µg		42	0.8	41	1.6	97.6

[a] These known mutagens, along with phenol (PN) and hexadecane (HPN), were mixed together and fractionated utilizing the fractionation scheme (Figure 2). Assays were performed on all chemical classes with strains TA1535, TA1537, TA1538, TA98, and TA100, with and without metabolic activations. Phenol and hexadecane were not detectable as mutagens in the Ames/*Salmonella* assay but were utilized as representative of their respective chemical classes. 9-Aminoacridine was detected with TA1537 without S9, 20-methylchloranthrene was detected with TA98 and S9. 5-Nitrofuroic acid was not detectable due to toxicity to *Salmonella* strain TA1535. Only positive results were reported here; chemicals were therefore strain- and class-specific, with activity qualitatively similar before and after fractionation. Spillover of the base compounds (9AA) into other chemical classes was evidenced by reduced mutagenic activity at 100 µg after fractionation; data suggests spillover of 9AA (base) into other classes.

[b] Mutagenic.

[c] Required Aroclor-induced S9 fraction (6.0 mg protein per plate).

[d] Toxic response.

[e] Toxic response before fractionation - percent recovery could not be calculated.

either with or without metabolic activation. Consequently, although spillover among chemical-class fractions probably did occur, it was not biologically detectable under the conditions tested. This experiment suggested that the fractionation scheme was capable of retaining mutagenic activity in a strain-specific, chemical-class-specific and activation-specific manner.

These evaluations suggested that the developed chemical fractionation scheme could give high recoveries of known mutagenic and nonmutagenic compounds and could qualitatively detect known mutagenic compounds in their proper fractions, with the proper strain and under the proper activation conditions. However, spillover between chemical-classes (e.g. base fraction) was detected and additional research is necessary to clarify the resolution ability of the fractionation scheme.

Methods for Chemical Identification of Compounds in Chemical-Class Fractions From Ambient Air Particles

For identification of the components in each chemical fractions, a GC/MS/Computer system was employed (Bursey et al., 1977). The GC/MS studies were performed with an LKB 2091 gas chromatograph/mass spectrometer. The samples were chromatographed on a 25m SE-30 capillary column ($BaCO_3$-treated wall) using a linear temperature program. The column was held at 100°C for two min after injection, and then heated to 240°C at a rate of 8°/min. Carrier gas flow rate was approximately 2.0 ml/min with an injection split ratio of 10:1. Injector temperature was 240°C. Scanning cycle times were approximately two sec, with a range of m/z from 50-492. In order to identify the overall sample component profiles, total ion current plots and mass spectra were generated. The plotted mass spectra were manually interpreted by a skilled interpretor and compared to those spectra compiled in a data compendium. In general, a minimum match of five masses and intensities (±15%) were obtained between the unknown and library spectrum. Identifications were achieved by comparison of mass spectral data with the *Aldermaston Eight Peak Index of Mass Spectra* (Mass Spectrometry Data Centre, Aldermaston, Reading, United Kingdom, Vol. III, 1974). Chemical identification methods were similar for both particulate organic matter and vapor-phase organic compounds. A more detailed discussion of the operating parameters for the GC/MS/Computer system is given in Appendix A.

Development of Biological Procedures to Test the Mutagenicity of Ambient Air Particles

The mutagenicity bioassay employed in this study was the *Salmonella* mammalian microsome assay developed by Ames et al. (1973, 1975a,b) and Ames (1979) with certain modifications (see Appendix B and C). The assay is based on reversion of histidine-requiring auxotrophs to the wild-type upon addition of a mutagenic substance. Five *Salmonella typhimurium* strains were used. Strains TA100 and TA1535 detect base-pair substitution mutagens while TA1537, TA1538, and TA98 detect frameshift mutagens. The addition of the R plasmid in strains TA98 and TA100 increases overall mutagen sensitivity detection but decreases the ability of the strains to detect specific types of mutagens (Ames et al., 1975b). Promutagens were activated with an Aroclor 1254-induced rat liver S9 microsomal preparation, which was added to the basic test system (Ames et al., 1973 and 1975b). If the samples were not toxic to the *Salmonella* strains, mutagenic activity was proportional to the number of colonies produced upon addition of sample and a 48-72 hr incubation period at 37°C.

The assay protocols utilized were the standard plate incorporation technique, the spot test (Ames et al., 1975b; Appendix B), and the agar well diffusion test (Hughes et al., 1980; Appendix C) which was developed under this program. The assay technique utilized depended upon sample size. A bioassay priority scheme based on sample size was developed to maximize the information per unit sample (see Table 4). When sufficient sample was available for bioassay purposes (greater than 10 mg), the Ames assay was performed with the standard plate incorporation technique. A mutagenic response for the Ames plate incorporation test was considered positive if the increase in colonies observed was twice the background rate and increased as a function of dose. Since sample size was limited in this study, only two doses of each fraction were tested. Therefore, the second criteria for a positive mutagenic response was not met. Samples were therefore considered potentially mutagenic with the plate incorporation technique if one dose produced an increase in colonies that was twice the background rate. Standard protocols for the plate incorporation and spot test technique of the Ames/*Salmonella* assay are contained in Appendix B.

The other assay technique utilized and developed under this program was the agar well diffusion test, which was useful for initial evaluation of mutagenic potential when sample size was less than 10 mg. The agar well diffusion test permitted *qualitative* determinations of direct-acting mutagens, promutagens, and toxicity on the same plate, which greatly optimized the amount of information that could be obtained for a limited sample size (10 mg or less). The major disadvantage of the well test was that mutagenic compounds of low aqueous-solubility [*e.g.*, benzo(a)pyrene] could not be detected due to the inability of these hydrophobic compounds to migrate into the aqueous based agar medium. The method-of-choice was therefore the standard plate incorporation technique when sufficient sample was available (greater than 10 mg of organic extract). The methods, utility, and criteria for a positive response for the agar well diffusion test are detailed in Appendix C and are briefly outlined below.

The agar well assay was developed and utilized in this developmental program because sample size was a critical limiting factor. For example, a minimum sample requirement of 178 mg has been established by the EPA for initial mutagenesis testing (Duke, 1977). Furthermore, Teranishi et al. (1978) and Tokiwa et al. (1977) required 100-2000 μg of air particulate extract per plate to produce a dose related mutagenic response. Significant mutagenicity required at least 250 μg of air particulate extract per plate. The Battelle® Massive Air Volume Sampler (MAVS) developed by Henry and Mitchell (1978), which was utilized during this

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TABLE 4. BIOASSAY PRIORITY SCHEME

(1) Sample amount >10 mg
Plate incorporation technique was utilized at five dose levels. The S9 (Aroclor-induced) concentration was 3.0 mg of protein/plate. Duplicate plates for each strain were used in the following order: TA98, TA100, TA1535, TA1537 and TA1538. Toxicity testing was performed at highest dose level only. Preferred initial dose ranges were 1,000, 500, 100, 10 and 1 µg/plate, S9 addition first, then no S9.

(2) Sample amount <10 mg
Agar well diffusion test was utilized at two concentrations (i.e., 500 and 100 µg/plate) except for PNA fractions which were tested with plate incorporation assay at 500 µg/plate, Aroclor-induced S9 and TA98. Duplicate plates were tested in the following order: TA98, TA1535, TA1537, TA1538 and TA100. Sample was diluted to one concentration for all tests; to obtain lower test concentrations, less sample volume per well was used. Toxicity testing was performed at 500 µg/plate with TA98 and TA1535 with 1,000 colonies/plate.

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study, generated only 1-4 g of test material during a ten day collection period. Since 5-10% of this material was organic, only 50-450 mg of sample was available for fractionation, chemical identification, and mutagenesis testing. This total sample amount resulted in most chemical-class fractions having less than 10 mg of extract for mutagenicity testing and chemical identification. Consequently, a modified assay technique for the Ames/*Salmonella* assay was necessary which could provide more information per unit sample.

The agar well diffusion test (Hughes et al., 1979; Pellizzari et al., 1979; Hughes et al., 1980), is a modification of the spot test (Ames et al., 1975) and was based on the principle of diffusion of proteins in agar first developed by Ouchterlony (1968). The technique utilized agar wells in the base layer to hold sample and S9. The agar well diffusion assay is a qualitative assay. Results are read as follows: *direct-acting mutagen*, colonies around or in sample well; *promutagen*, crescent-shaped line of colonies between the sample and S9 wells; and *toxicity*, clear "kill" zone around the sample well (see Appendix C for complete procedure). The agar well test reduced the amount of sample required for initial analysis, since direct-acting mutagens, promutagens, and toxicity were all detected on a single plate. The agar well diffusion test was designed for situations where sample size was less than 10 mg (see Table 4; Bioassay Priority Scheme). Criteria for a positive mutagenic response in both the spot test and agar well diffusion technique were visual and were therefore qualitative. A significant increase in the number of colonies in the vicinity of the agar well or disc (in the spot test) was considered a positive response. Methodology for the spot test is contained in Appendix B and for the agar well test in Appendix C.

False negative results may be generated in the agar well test due to a pH effect, low diffusion rate, or toxicity of the sample to the bacteria or S9. However, this test has aided the development of an efficient solvent fractionation scheme and enabled the identification of mutagens within specific chemical-classes, each containing hundreds of compounds (Pellizzari et al., 1979). The agar well test was similar in sensitivity to the spot test, but it offered the advantage of qualitatively testing multiple parameters (dose range finding, activation, toxicity, and mutagenicity) on a single plate. This ability expanded the information obtainable from a limited sample. The use of the agar well diffusion assay when sample size was minimal (<10 mg) was a distinct advantage in this air pollution study.

VAPOR-PHASE ORGANICS (VPOs)

Methods for Sampling and Chemical Identification of Vapor-Phase Organics

Vapor samples were collected on Texas GC polymeric sorbent. Cartridges containing $\sim$20 g of sorbent were used to collect vapors for subsequent desorption and mutagenesis testing. Parallel sampling was carried out with smaller cartridges ($\sim$1.5 g Texas GC) in order to collect vapor-phase material for chemical characterization studies. For the smaller cartridges, up to 100 ℓ of ambient air was sampled. The cartridges were returned to the laboratory and analyzed by thermal desorption/GC/MS/COMP analyses (Pellizzari et al., 1975; Pellizzari, 1979). The collection and analysis systems are graphically shown in Figures 4 and 5. The general method of sampling and analysis are outlined below; quality control data for this system is contained in Appendix A.

The analytical sampling tubes were prepared by packing a 10 cm long by 1.5 cm i.d. glass tube containing 6 cm of 35/60 mesh Tenax® GC with glass wool in the ends to provide support (Pellizzari, 1975; Pellizzari, 1977). Virgin Tenax® was extracted in a Soxhlet apparatus with acetone and hexane for 18 hr immediately prior to preparation of cartridge samplers. After purification of the Tenax® GC sorbent and drying in a vacuum oven at 100°C for 3-5 hr at 28 inches of water, all the sorbent material was meshed to provide a 35/60 particle size range. Cartridge samplers were then prepared and conditioned at 270°C with helium flow at 30 ml/min for 30 min. The conditioned cartridges were transferred to Kimax® (2.5 cm x 150 cm) culture tubes, immediately sealed using teflon-lined caps and cooled. This procedure was performed in order to avoid recontamination of the sorbent bed. These sampling tubes were then utilized for field sampling. A sample tube which was not opened was utilized as a control.

Sampling periods employed in the field were from 1-3 hr. Duplicate Tenax® GC sampling cartridges were employed. The flow rate was maintained at a rate of 25-100 ml/min. Personal monitoring samplers (MSA, Model C-200), were utilized for collection. The samples collected on Tenax® GC cartridges were anlyzed by capillary column GC/MS/Computer. The whole sample was introduced to the analytical system by thermal desorption into a cryogenic capillary trap and thence rapidly swept into the chromatographic column. Each cartridge was loaded with a known amount of perfluorobenzene and perfluorotoluene for use in quantitation. Qualitative data interpretation was done by visual interpretation of the spectra and comparison with standard reference spectra. Quantitation was acheived by integration of a characteristic mass for a compound over the chromatographic peak. The amount of compound in the sample was then calculated using the relative molar response factor and the integrated area for a standard. The concentration in ambient air was then calculated with the volume sampled or the breakthrough volume, whichever was larger. Complete details of the sample analysis are in Appendix A.

The large sorbent cartridges (~1000-3000 ℓ of air sampled) we treated in essentially the same fashion as the smaller analytical cartridges. This method involved desorption of volatile components from the Tenax® cartridge at a high temperature (225°C) with flowing helium (Figure 6). The desorbed material passed through the 3-way valve (position AB) to a coiled 1 m length of Teflon® tubing (1.35 mm i.d.), which was partially immersed in liquid nitrogen (LN$_2$). Purging was continued for 45 min to ensure complete removal and entrapment of the volatiles. At the end of this period the LN$_2$ bath was replaced by an ice/H$_2$O bath. After equilibration (~5 min), the 3-way valve was manipulated (position BC) to stop helium flow and to bring the syringe in line. Small amounts

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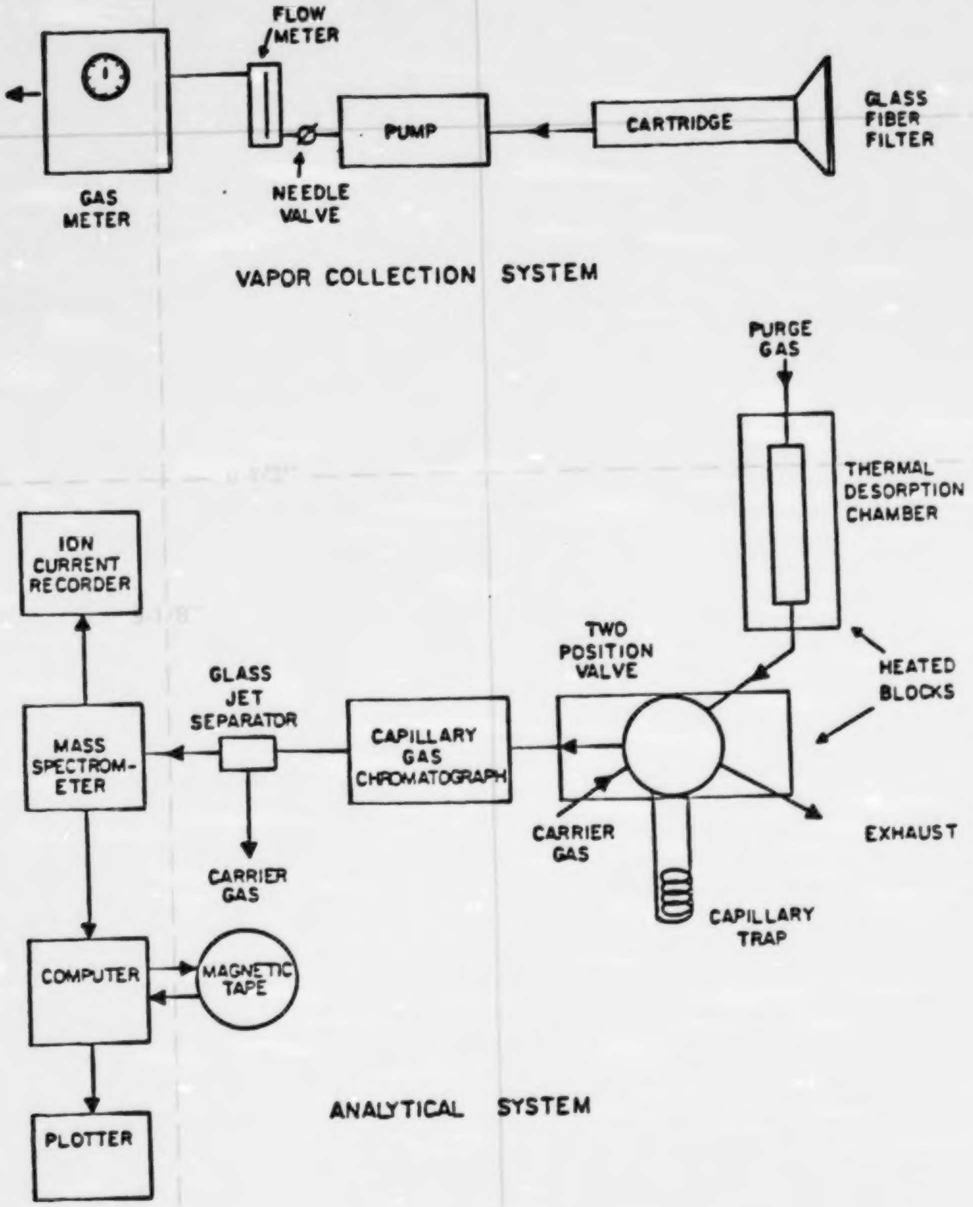
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ION CURRENT RECORDER
TWO POSITION VALVE
HEATED BLOCKS
GLASS JET SEPARATOR
MASS SPECTROMETER
CAPILLARY GAS CHROMATOGRAPH
CARRIER GAS
EXHAUST
CARRIER GAS
CAPILLARY TRAP
COMPUTER
MAGNETIC TAPE
PLOTTER
ANALYTICAL SYSTEM

Figure 4. Vapor collection and analytical systems for analysis of organic vapors in ambient air.

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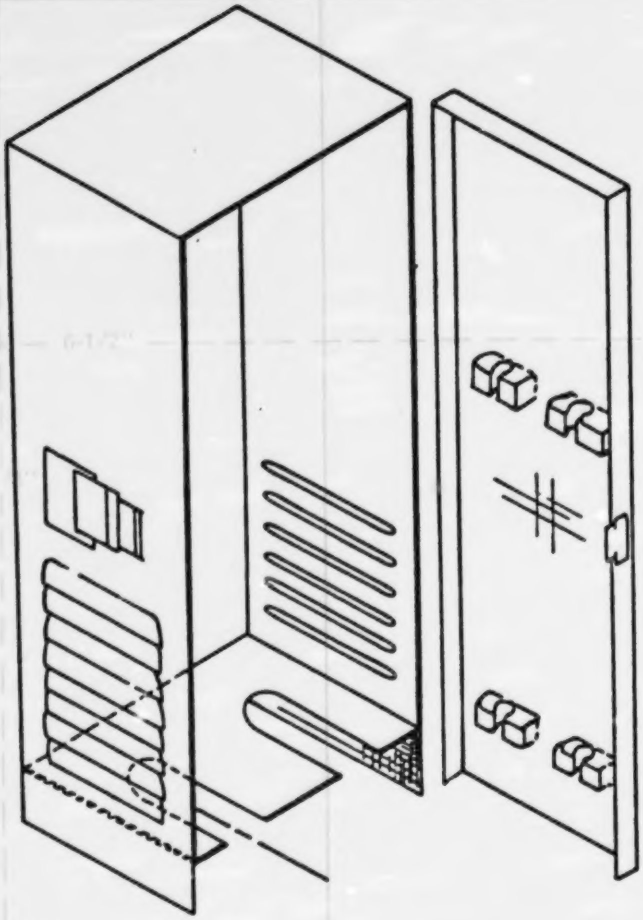

Figure 5. Sampling head cover for cartridge sampling train.

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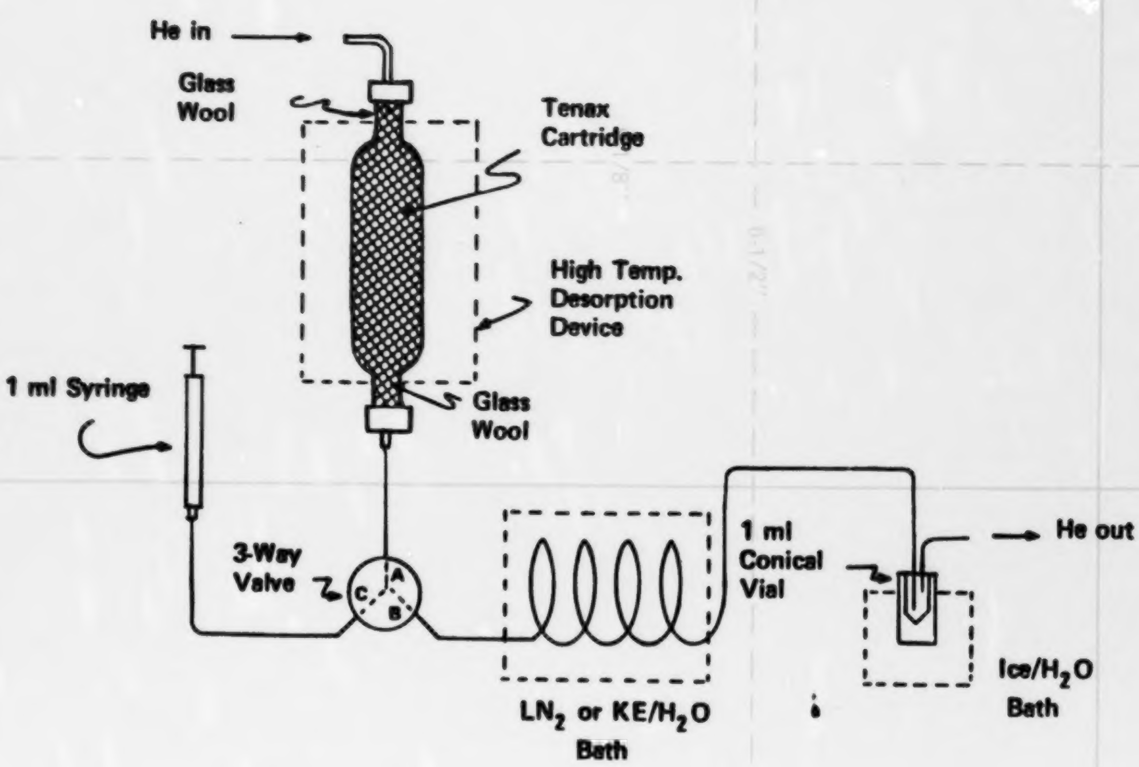
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1 ml Syringe
3-Way Valve
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C
LN_2 or KE/H_2O Bath
1 ml Conical Vial
He out
Ice/H_2O Bath

Figure 6. Schematic of thermal desorption/vapor collection device.

(usually 0.25 ml) of ethylene glycol was discharged from the syringe through the coil to dissolve the trapped components. The ethylene glycol solution was collected in a 1 ml vial, which was immersed in an ice/H_2O bath. The volatiles were thus concentrated in the solvent.

Validation studies with regard to transfer efficiency of this setup were performed. In these studies a known amount of radiolabeled acetone or toluene (Figures 7 and 8) was loaded onto a clean Tenax® cartridge. Toluene was representative of the upper end of the volatility range (bp 111°C). Successful desorption of this material would suggest that most adsorbed volatiles would be amenable to the desorption procedure just described. The cartridge was therefore desorbed and the amount of collected compound was determined by liquid scintillation counting.

The loading of a clean cartridge was accomplished by vaporization at 230°C with varying quantities (~150-~900 μg) of each component in an inert gas stream flowing through a Tenax® cartridge. The outlet gas stream was monitored for sample breakthrough by passage of the gas through two tandem solutions of scintillation cocktail. No breakthrough was noted for either compound at any time. The cartridges were then desorbed with the apparatus illustrated in Figure 6. Three different sample sizes of toluene and acetone (0.2 μl, 0.5 μl, and 1.0 μl) were tested in triplicate. During the desorption phase, the helium outlet was passed through scintillation fluid to trap any material not successfully collected in the Teflon® coil. After the desorption phase was completed, several aliquots (100 μl) of ethylene glycol were passed through the coil and collected separately for direct radioactivity determination. Five discrete 100 μl volumes of ethylene glycol solution were collected serially for final counting. The results are depicted in Figures 7 and 8. The radioactivity (dpm's) were normalized to 100% (i.e., to the amount loaded onto each cartridge). The first vial (designated "B") represented the amount of each compound that passed through the liquid nitrogen trap during the desorption/collection phase. The remaining five vials (numbers 1-5) represent the amount of ^{14}C-labeled compound collected by each of five 100 μl aliquots of ethylene glycol used to flush the collection coil.

The recovery of acetone at all levels was quantitative (Figure 74). No breakthrough from the liquid nitrogen trap was observed, and it appeared that flushing with 200 μl of ethylene glycol was sufficient for at least 90% recovery of acetone, and by extension other compounds of similar volatility and structure. The overall recovery of toluene was also high (Figure 8), although larger volumes of flush liquid were required to achieve these high values compared to the acetone runs. There was a significant amount of toluene collected in the "breakthrough" vial prior to the ethylene glycol flush sequence for some runs. This may be the result of aerosol formation which could cause the toluene to pass through the liquid nitrogen trap. Recoveries, well in excess of 100%, were also noted for toluene at the 174 μg level; this was caused by the Tenax® cartridges, which were used repeatedly and were not subjected to a sufficiently high temperature or period of desorption regimen to desorb all of the compound. The nondesorbed material could then contribute

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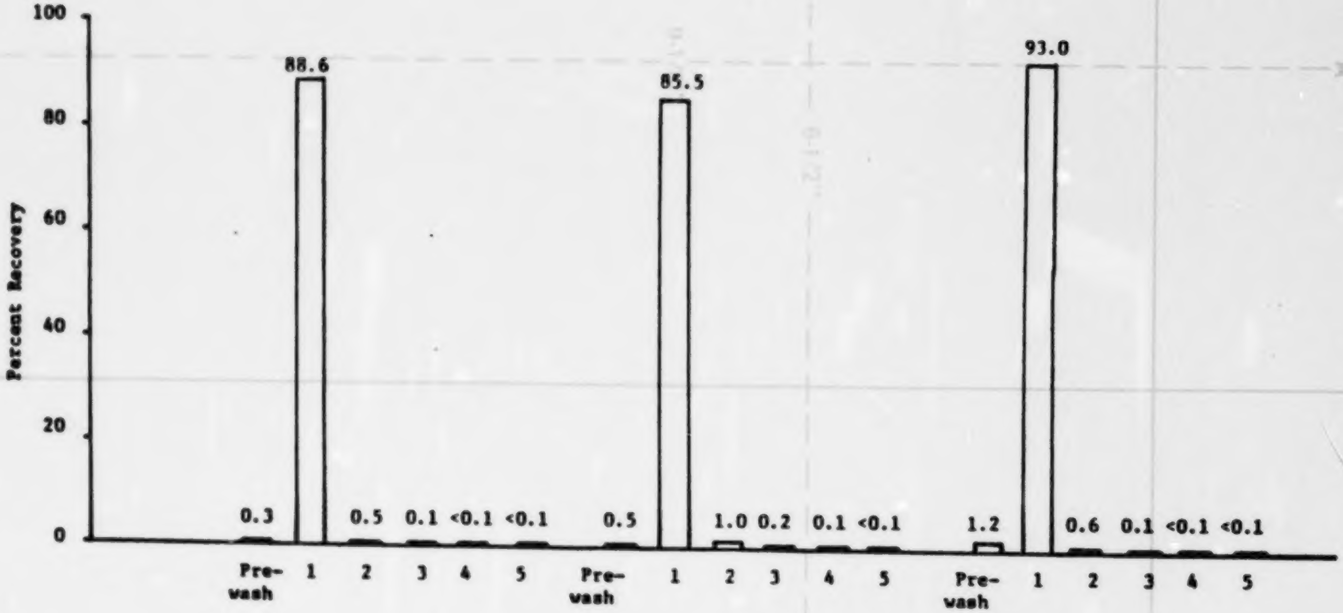

Figure 7. Recovery of ^{14}C-acetone (790 μg) from Tenax® cartridge by thermal desorption/cryogenic cooling. Fractions 1-5 for each sample represent serial solvent washes (100 μl ethylene glycol) of the trapped vapors.

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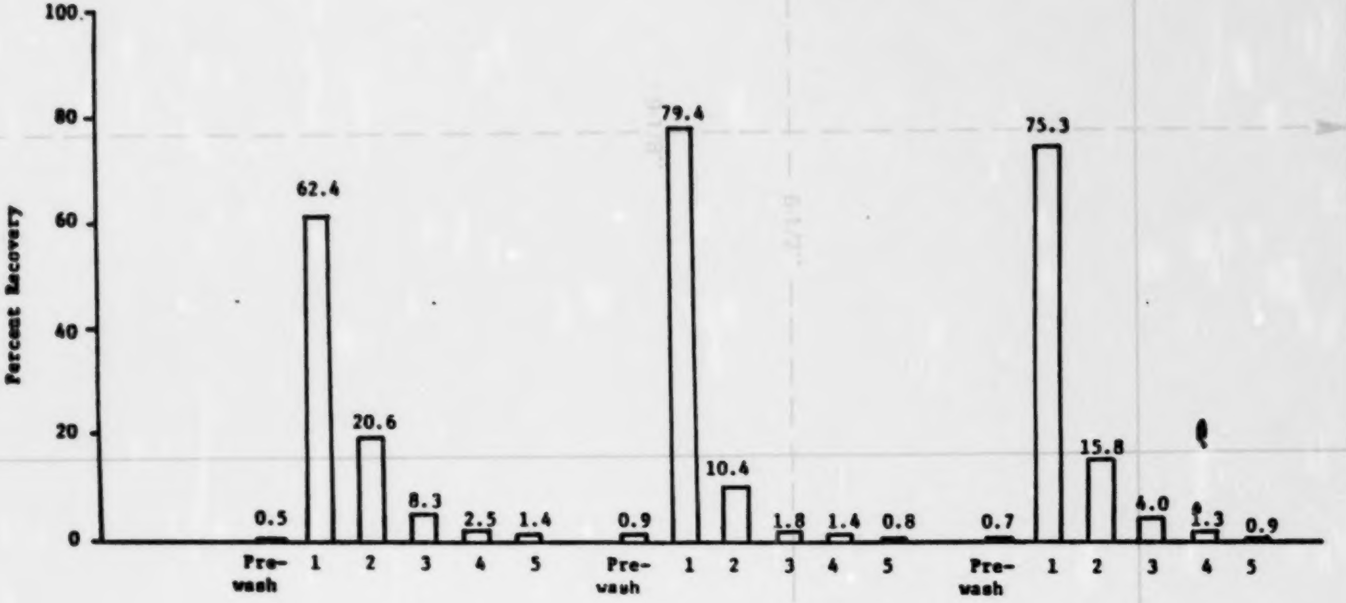

Figure 8. Recovery of ^{14}C-toluene (866 μg) from Tenax® cartridge by thermal desorption/cryogenic cooling. Fractions 1-5 for each sample represent serial solvent washes (100 μl ethylene glycol) of the trapped vapors.

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to the amounts collected when the cartridge was reused. This problem was corrected by purging the Tenax® at a higher temperature (250°C) for a longer time period. The vapors collected in the ethylene glycol solvent were then either stored in a -80°C refrigerator or immediately analyzed for chemical components with a GC/MS/Computer, as described earlier for the particle analysis.

Development of Techniques to Test the Mutagenicity of Ambient Vapor-Phase Organics

An adequate quantitative technique to measure vapor-phase organic mutagens in the Ames/*Salmonella* assay had not been developed when this program was initiated in 1977. Therefore, it was not possible to perform routine mutagenicity testing of vapor-phase organic field samples under this program. Initial studies by Malaveille et al. (1975,1976) detected mutagenicity of vinyl chloride to *Salmonella* strains TA1530, TA1535, G46, and TA100 at concentrations of 2 and 20% w/v in a dessicator. Rat liver, which was induced with phenobarbitone and stabilized with EDTA, was utilized as the metabolic activation system in these studies. A 2 hr preincubation of the bacteria with the S9 prior to a 3 hr exposure to the vinyl chloride was required to detect a mutagenic response. The S9 enzymatic potential was studied and was found to remain stable in soft agar for 7 hr. However, quantitation of this method was difficult because of the high volatility and aqueous-insolubility of vinyl chloride.

The standard Ames/*Salmonella* plate incorporation method (Ames et al., 1975b) was not adequate to detect the mutagenicity of compounds with high vapor pressure because this technique requires an initial exposure to agar at 45°C and incubation with sample in agar at 37°C. Substances which boil below this temperature will volatilize before the system has an opportunity to form mutagenic compounds. Rannug et al. (1976) have utilized preincubation of the *Salmonella* strains and S9 (1 hr at 25°C) to study the metabolites of vinyl chloride, and have demonstrated that chloroethylene oxide and chloroacetaldehyde are the major metabolites. Quantitation was difficult in this study because of the low aqueous-solubility and high volatility of vinyl chloride. Rosenkranz et al. (1979) have used a modified spot test to detect vapors; discs impregnated with volatile sample were incubated at 4°C for 2 hr, removed, and incubated as usual (37°C for 48 hr), or the discs were placed in sealed plastic bags and incubated. A limitation of this method is that nondiffusible (normally aqueous-insoluble) test agents will not be detected, and quantitation will therefore be difficult.

Research was thus directed toward the development of a method that would encompass the efficient collection and storage of airborne vapor-phase compounds in such a fashion that would permit subsequent analysis of the compounds with an Ames test system. This method should permit the quantitative biological and chemical evaluation of the mutagenicity of volatile, nonaqueous, vapor-phase organics in the Ames/*Salmonella* mammalian microsome mutagenicity assay (Ames et al., 1975b). Ideally, the developed technique would allow for optimal diffusion of the vapor-phase organic sample in a test system with minimal dead air volume;

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would allow for optimal association of the bacteria, S9 metabolic activation system and the test compound; and would demonstrate increased mutagenic activity of volatile vapor-phase organics over that detected in the standard pour plate assay (McCann et al., 1975). In addition, this technique would be compatible with vapor-phase organic field samples that had been collected on Tenax® cartridges, thermally desorbed and cryogenically frozen in ethylene glycol.

It was immediately obvious that a major obstacle to introduction of vapors and gases into the standard Ames/Salmonella assay was physical containment of these materials, and quantitative exposure of the vapor-phase organic compounds to the bacteria and S9 metabolic activation system on the agar plate. Initial experiments assessed granular charcoal as a method to contain vapor-phase organics. It was envisioned that organic vapor-phase compounds could be sampled and trapped on the charcoal, with subsequent slow release of the sample into the agar with the use of solvents and a 37°C incubation temperature. However, charcoal was eliminated from consideration after it was demonstrated that 2-anthramine could not be desorbed from the charcoal with DMSO. The type of charcoal utilized in this preliminary study was a mesh that had previously been utilized to trap vapor-phase organics in ambient air field studies (Pellizzari, 1977).

Subsequently, hollow-fiber filter tubing (HFT) and polyethylene tubing (PET) (Romicon Corporation, Woburn, MA) were investigated as possible vehicles for physical containment and exposure of vapor-phase organics. These materials were chosen based on their high degree of permeability to low molecular weight compounds. Hollow-fiber filters, with nominal porosities from 2,000-50,000 MW, were tested with different solvents (DMSO and ethylene glycol) and a variety of known mutagens. The fibers (1.1 mm i.d. and 6 cm in length) were filled with the test material, sealed by crimping the ends with hot tweezers, placed on top of a base agar layer, and then covered with an overlay which contained the bacterial tester strain (and S9 metabolic activation system when appropriate). Since initial studies with sodium azide were promising, further experiments were conducted with 9-aminoacridine, benzo(a)pyrene, 8-aminoquinoline, benzathracene, and 2-anthramine. Three 6 cm fiber segments were utilized per plate and were placed in the agar plate, in the form of a triangle. Each segment contained 40 μl of solvent, with or without test compound. Positive results were obtained only with 9-aminoacridine (Table 5). These results suggested that the other compounds either failed to diffuse into the aqueous base agar or did not diffuse from the hollow-fiber filter tubing. Investigations into solvent addition to molten agar (45°C) were attempted to increase the diffusivity of these aqueous-insoluble substances. Consequently, 100 μl of DMSO was added to molten agar (45°C) and the agar was allowed to solidify. Subsequent addition of tubing with the above known mutagens to DMSO-enriched agar did not increase migration distances as measured by the distribution of revertant colonies (see Table 6).

Upon completion of the preliminary experimentation with the hollow fiber filter technique and its resultant limitations, it became evident

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TABLE 5. HOLLOW FIBER EXPERIMENTS WITH A VARIETY OF TEST COMPOUNDS

Fiber Type	Solvent	Test Compound	Concentration µg/fiber	Test Strain	S9 Addition	Colonies/plate Plate 1	Colonies/plate Plate 2
PM-2	DMSO	9-aminoacridine	80	TA1537	-	701[a]	601[a]
		8-aminoquinoline	4	TA1537	+	29	33
		8-aminoquinoline	4	TA1537	-	32	45
		benzanthracene	20	TA98	+	59	39
		benzanthracene	20	TA98	-	35	38
		benzo(a)pyrene	4	TA98	+	4	53
		benzo(a)pyrene	4	TA98	-	41	55
		2-anthramine	40	TA98	+	55	41
	DMSO	-	-	TA98	+	39	41
		-	-	TA98	-	21	29
		-	-	TA1537	-	14	-

[a] Mutagenic response.

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TABLE 6. INITIAL VAPOR VALIDATION RESULTS WITH SALMONELLA STRAIN TA100

Sample	Conc. Sample µg/plate	Fiber with DMSO[a] (MR)[b]	Fiber without DMSO (MR)	Standard Pour Plate (MR)	Preincubation Technique[c] (MR)
Sodium	200 + S-9	10.5	10.8	10.4	9.8
Azide	20 + S-9	7.7	7.1	9.7	8.7
	2 + S-9	1.8	2.0	1.5	1.2
	200 without S-9	7.7	2.0	7.4	6.7
	20 without S-9	6.3	1.1	8.0	6.4
Anthramine	100 + S-9	3.7	3.2	5.2	8.6
	10 + S-9	2.5	2.0	13.8	9.4
	1 + S-9	1.1	1.3	4.4	5.3
Controls	+ S-9	139	136	171	170
(MR = 1.0)	- S-9	180	206	213	228

[a] 100 µl of DMSO was added to base layer agar at 45°C and was allowed to solidify before addition of fiber with the mutagenic sample.

[b] MR = Mutagenic Ratio = $\frac{\text{Revertants with Sample}}{\text{Spontaneous Revertants}}$ (average of two plates).

[c] Sodium azide and 2-anthramine were incubated at 4°C for 3 hr and then at 37°C for 45 min in sealed vials (without fibers) with TA100.

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that alternate techniques for detection of mutagenic vapors must be investigated. A literature review identified preincubation of high vapor-pressure compounds with bacteria and metabolic activation systems in liquid suspension as an effective method for detection of vapor-phase mutagens (Milvy and Garro, 1976). Consequently, mutagens were added directly to liquid suspension of bacteria. For comparison, results from the standard plate incorporation technique and the hollow-fiber filter technique (with and without DMSO addition to the agar base layer) were compared (Table 6). Results suggested a significant decrease in mutagenic activity with the hollow-fiber filter technique when it was compared to the standard pour plate technique (Table 6), especially in the case of the aqueous-insoluble compound 2-anthramine. These solubility limitations caused further research with the hollow-fiber filter technique to be discontinued. However, similar mutagenic activity was demonstrated in the pour and preincubation assays, for both sodium azide and 2-anthramine.

The previous experiment led to the development and utilization of a modified preincubation technique, as initially developed by Yahagi et al. (1975) for the detection of vapor-phase mutagens. It was demonstrated in the above experiment that preincubation would detect known nonvolatile positive mutagenic compounds (both direct- and indirect-acting mutagens) at a sensitivity level that was comparable with the standard plate incorporation technique. Furthermore, a liquid suspension preincubation assay would allow for optimal association of bacteria, S9, and test compound, would be compatible with cryogenically frozen, Tenax®-desorbed samples, and would be applicable to promutagens (Malaveille et al., 1975; Yahagi et al., 1975). Incubation at 37°C would volatilize low boiling point vapor-phase organics in a closed system with no dead air volume, which would increase the association of the test compound with the bacteria. For this reason, incubation at 37°C was utilized, rather than incubation at 0° or 4°C.

Since the methodology utilized for collection and chemical analysis of VPOs had been defined (Appendix A), this concept was broadened to allow for the collection of VPOs in amounts sufficient for bioassays. Thus, cartridges with approximately 10 times as much Tenax® as resin the analytical cartridges were employed to collect field samples.

Research was then directed toward development of procedures for efficient removal and collection of the trapped VPOs from the Tenax®. Thermal desorption was chosen as the preferred method for stripping VPOs from the sorbent since; (1) it is highly efficient; and (2) it precluded solvent desorption (a more tedious and less efficient stripping procedure).

The apparatus used for thermal desorption and collection of the VPOs from Tenax® is shown in Figure 6. It consisted only of glass, Teflon®, and stainless steel, and was constructed to minimize dead volume. This desorption procedure concentrated the volatile compounds into solvent at cryogenic temperatures. Cryogenically frozen samples could then be incorporated into the Ames/Salmonella assay with the developed preincubation technique. Mutagenic field testing of vapor-phase

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organics was not accomplished under this program but chemical identification of signature mutagens in vapor-phase field samples was performed with these procedures. Results of the chemical analysis are in Section 5.

The final preincubation protocol developed under this program is outlined in Table 7. This protocol was utilized to test pure vapor-phase compounds found in ambient air. Results are described in Section 5. This technique was modified from the technique of Yahagi et al. (1975) to increase sensitivity. These modifications included addition of top layer agar to the incubation vial to: (1) fill the dram vial dead air space; (2) provide a substrate for entrapment of volatile compounds; (3) provide trace amounts of histidine and biotin (0.04 mM) for replication of the bacteria during preincubation; and (4) provide for S9 stabilization, since Malaveille and co-workers (1976) had demonstrated that S9 was active in agar for 7 hr when vinylidene chloride was tested with Salmonella strain TA100. Log-phase cells were utilized to increase sensitivity. Guttenplan et al. (1977), had demonstrated a 3-fold increase in mutation rate in the Salmonella assay by the utilization of log-phase cells versus stationary-phase cells. Preincubation time was also investigated; optimal incubation time was 30 min at 37°C. Experiments conducted to optimize the sensitivity of the preincubation technique are discussed below.

Figure 9 depicts the experiment that determined the optimal mutational frequency with different growth phases of Salmonella. The number of cells per vial for both log and stationary grown cells was normalized since log-phase cultures contained less cells per volume than did stationary grown cells. This manipulation allowed the determination of mutational frequency per 10^6 viable cells. The study was conducted with Salmonella strains TA1535, TA100, and sodium azide; water was the solvent. Log-phase cells were more sensitive to sodium azide, and sensitivity was increased overall by a factor of three with the log-phase cells for both TA1535 and TA100. These initial results suggested that the use of log-phase cells was advantageous, since log-phase cells increased sensitivity, especially at lower doses of mutagens. This experiment was performed with the standard pour plate technique. Similar experiments were performed with the preincubation technique with TA1535, TA100, and sodium azide. Figures 10 and 11 demonstrate that sensitivity to sodium azide for these strains was increased with the preincubation technique compared to the standard pour plate technique. The optimal combination for the detection of mutagenic activity was therefore log-phase Salmonella cells and the preincubation technique.

The effect of incubation time in the preincubation assay was also investigated (Figure 12). Benzo(a)pyrene (BAP) was preincubated for 30, 60, and 180 min; vials either contained histidine/biotin agar or contained no agar. This experiment demonstrated that: (1) the optimal preincubation time was 30 min, since a linear decrease in mutagenic activity was detected with increasing incubation time beyond 30 min; (2) the addition of histidine/biotin agar to the preincubation vial increased mutational activity; and (3) the addition of histidine/biotin agar did not increase

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TABLE 7. PREINCUBATION TECHNIQUE PROTOCOL

1. *Salmonella* tester strains were grown overnight in 50 ml of Oxoid nutrient broth at 37°C with shaking. This was a stationary-phase culture.

2. This stationary-phase culture was then inoculated into 50 ml of fresh nutrient broth to attain log-phase cells. The amount of stationary-cells that was initially added to attain a starting O.D. of 0.5 at 420 mm was calculated according to the following formula:

$$\frac{\text{O.D. desired}}{\text{O.D. present}} \times 50 \text{ ml} = \text{amount of stationary cells to be added to nutrient broth.}$$

e.g., $$\frac{0.5 \text{ (at 420 nm)}}{2.0} \times 50 = 12.5 \text{ ml}$$

This starter culture was then grown for four hr at 37°C with shaking to attain the log-phase cells. Optical densities were always read at 420 nm; OD desired in the above equation was always 0.5. Final optical densities of log-phase cultures were between 1.7 and 1.9 at 420 nm.

3. The following components were added to a sterile 0.5 dram vial (with teflon lined caps) and mixed:

Without S9	
Agar[a]	600 µl
H_2O	100 µl
0.25 M Sucrose	300 µl
Log-phase cells	700 µl
Sample	300 µl
	2,000 µl/vial

With S9	
Agar[a]	600 µl
NADPH[b]	100 µl
S9[c]	300 µl
Log-phase cells	700 µl
Sample	300 µl
	2,000 µl/vial

[a] Regular histidine/biotin overlay agar.

[b] 9.6 mg NADPH/ml deionized-distilled H_2O.

[c] 30.0 mg protein/ml 0.25 M sucrose. Protein amounts were determined by Lowry method (Lowry et al., 1951). An additional 300 µl of S9 and 100 µl of NADPH was added to vials with S9 after preincubation. This additional S9 was added when the top agar layer poured. Therefore, 6 mg of S9 protein per plate was added for S9 studies.

4. These vials were then incubated at 37°C with shaking for 30 min.

5. After preincubation, 650 µl (in triplicate) was pipetted into a 2.5 ml histidine/biotin overlay and poured onto a base layer plate. Plates were inverted and incubated for 48-72 hr at 37°C.

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TABLE 7 (cont'd)

6. Revertant colonies were counted on an automatic counter; automatic counts were validated against hand counted plates.

7. Toxicity assays were conducted in tandem with the muatagenicity assays at each dose level.

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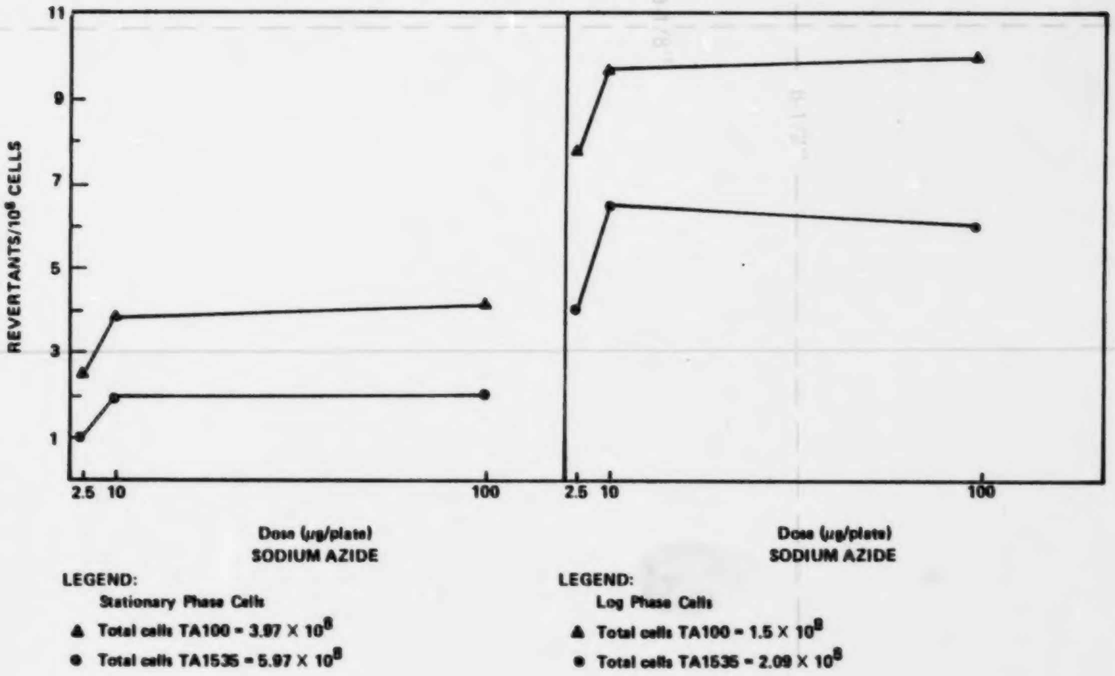

Figure 9. Mutagenicity of sodium azide with stationary- and log-phase cells of Salmonella strains TA100 and TA1535 (pour plate technique).

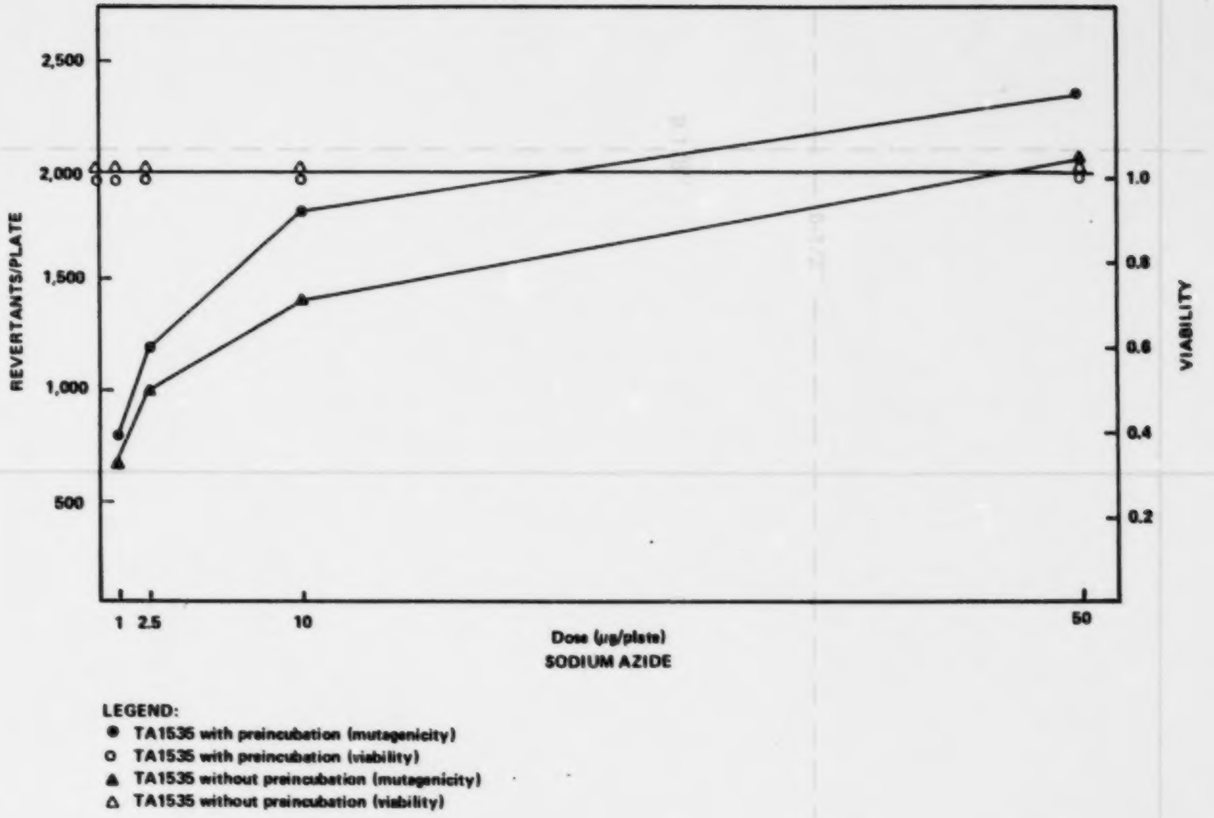

Figure 10. Mutagenicity of sodium azide with log-phase cells of *Salmonella* strain TA1535 (with and without preincubation).

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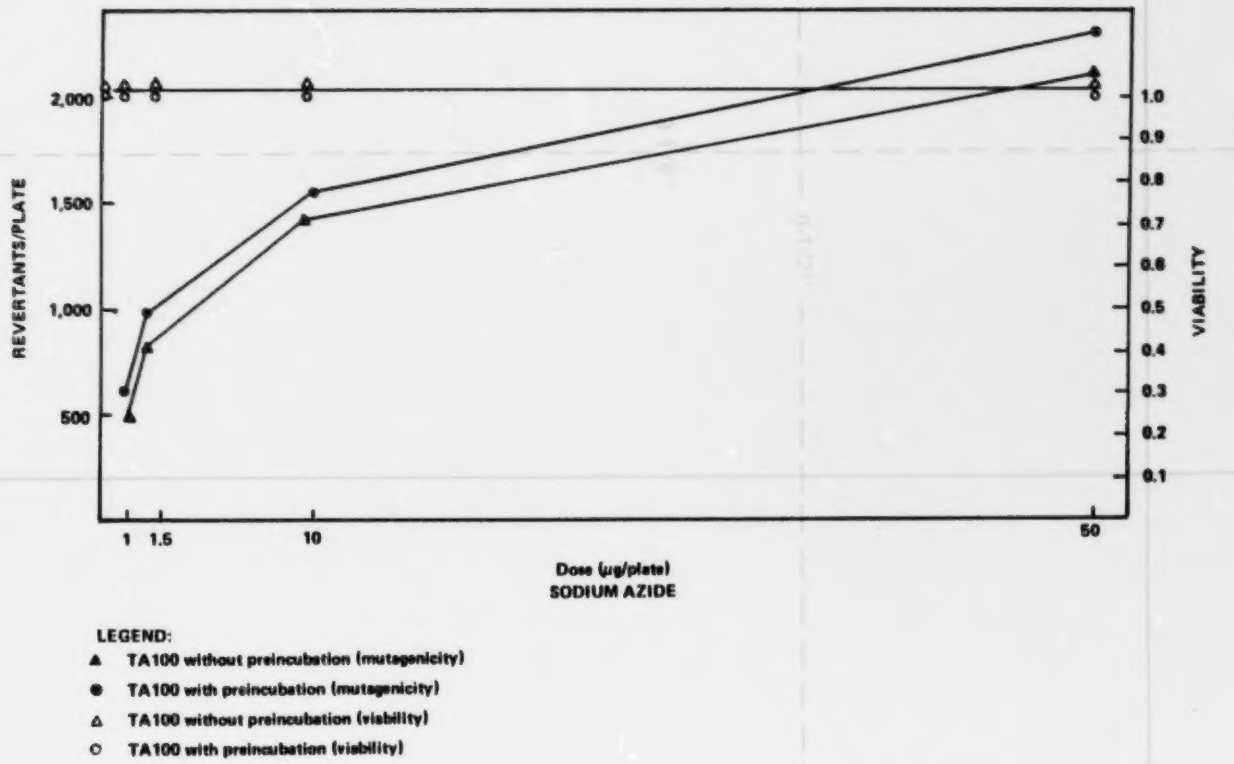

Figure 11. Mutagenicity of sodium azide with log-phase cells of Salmonella strain TA100 (with and without preincubation).

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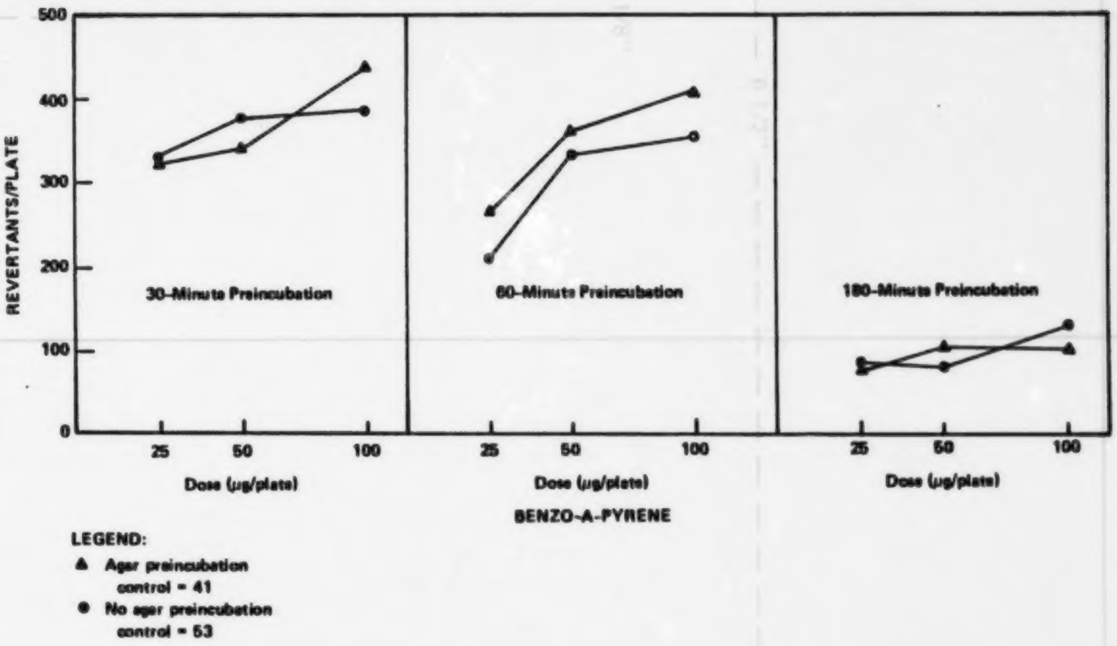

Figure 12. Effect of incubation time and the addition of histidine/biotin agar in the preincubation assay with Salmonella strain TA98 and benzo(a)pyrene.

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the spontaneous background rate, as the rate was 41 colonies with agar and 53 colonies without agar. Therefore, a 30 min incubation time with histidine/biotin top layer agar addition to the preincubation vial was utilized in all future validation experiments.

The addition of EDTA (ethylenediamine tetraacetic acid) to the S9 mixture was investigated to determine if the sensitivity of the preincubation technique could be increased by stabilization of the S9 extract in liquid culture. If such stabilization occurred, an increase in S9 microsomal mediated mutagenicity would be realized by an increase in mutagenic activity of a promutagen with time. Stabilization would not only increase sensitivity but would also increase the reproducibility of the assay. Experiments with EDTA, benzo(a)pyrene, preincubation, and different time intervals are shown in Figure 13. No significant increase in activity under any of the concentrations of EDTA (0.05, 0.25, and 1.0 µm) at any time point was demonstrated. S9 stabilization was also attempted with β-mercaptoethanol (BME) and dithiothreitol (DTT) in the preincubation technique. Benzo(a)pyrene (100, 50, 25 µg/plate) was added to vials which contained 0.5, 1.5, and 3.0 mM of either BME or DTT and preincubated at the same time intervals as in the above experiment. No significant increase in mutagenic activity was detected with either BME or DTT at any of the time points or concentrations tested.

Toxicity to the bacteria also increased with preincubation. This increase in toxicity to the bacteria was demonstrated in an experiment with 2-nitrofluorene, strain TA98, stationary- and log-phase grown cells, tested with and without preincubation (Figure 14). Toxicity of 2-nitrofluorene was greater with preincubation when compared to the standard pour plate technique. Toxicity also increased when results from log-phase cells were compared to stationary-phase cells. This graph was included to demonstrate that toxicity increased with preincubation. Althouth toxicity can cause the generation of false results by excessive killing of bacteria with subsequent release of histidine into the media, the increase in toxicity in the preincubation assay did indicate that the association of test compound with bacteria was also increased. The comparison of mutational frequencies in this experiment was not feasible, as the concentration of the log-phase cells was approximately half that of the stationary-phase cells (Figure 14). The addition of an equal volume of stationary- and log-phase cells/plate in this experiment resulted in fewer viable cells/plate with the log-phase cultures. This initial experiment consequently led to the addition of an increased number of log-phase cells/plate in future experiments, so that both stationary- and log-phase cells had an equal number of viable cells/plate.

Another parameter that was investigated in an attempt to increase the sensitivity of the preincubation assay was the sealing of the petri dishes with masking tape after the top agar layer had been poured. Similar mutagenic activity of ethylene oxide to *Salmonella* strain TA100 was demonstrated (Figure 15) whether or not masking tape was utilized. Consequently, the sealing of plates with masking tape was not performed.

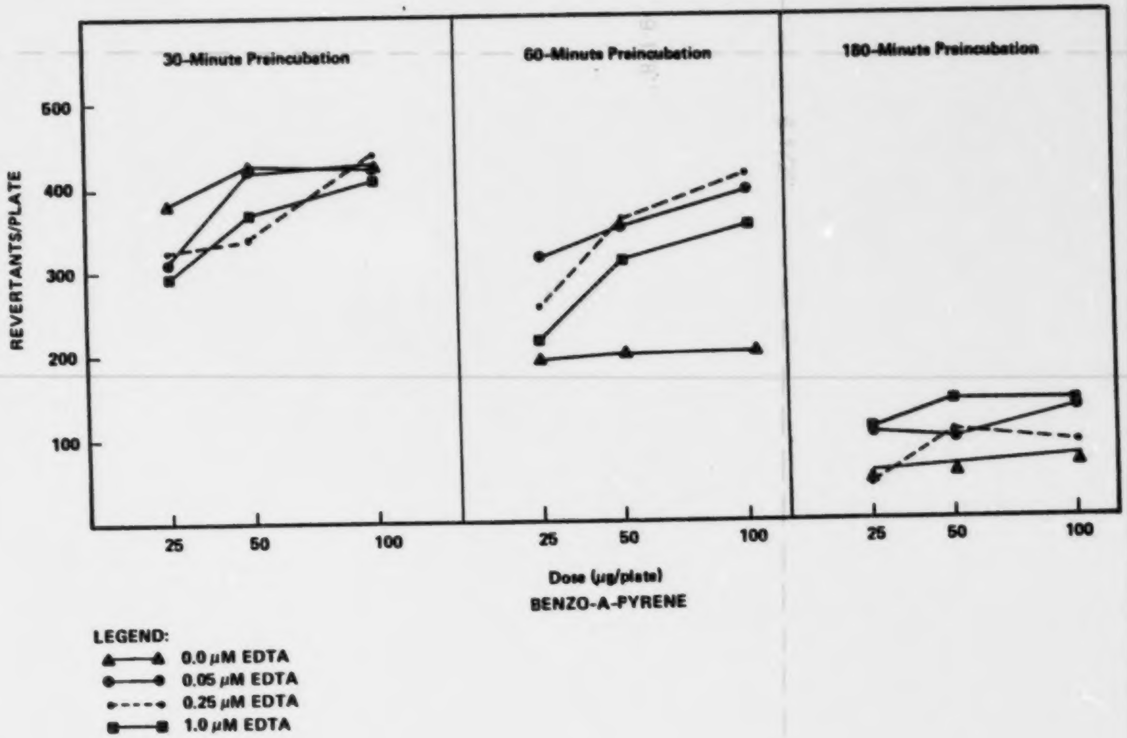

Figure 13. Effect of EDTA on benzo(a)pyrene mutagenicity in the preincubation assay with *Salmonella* strain TA98.

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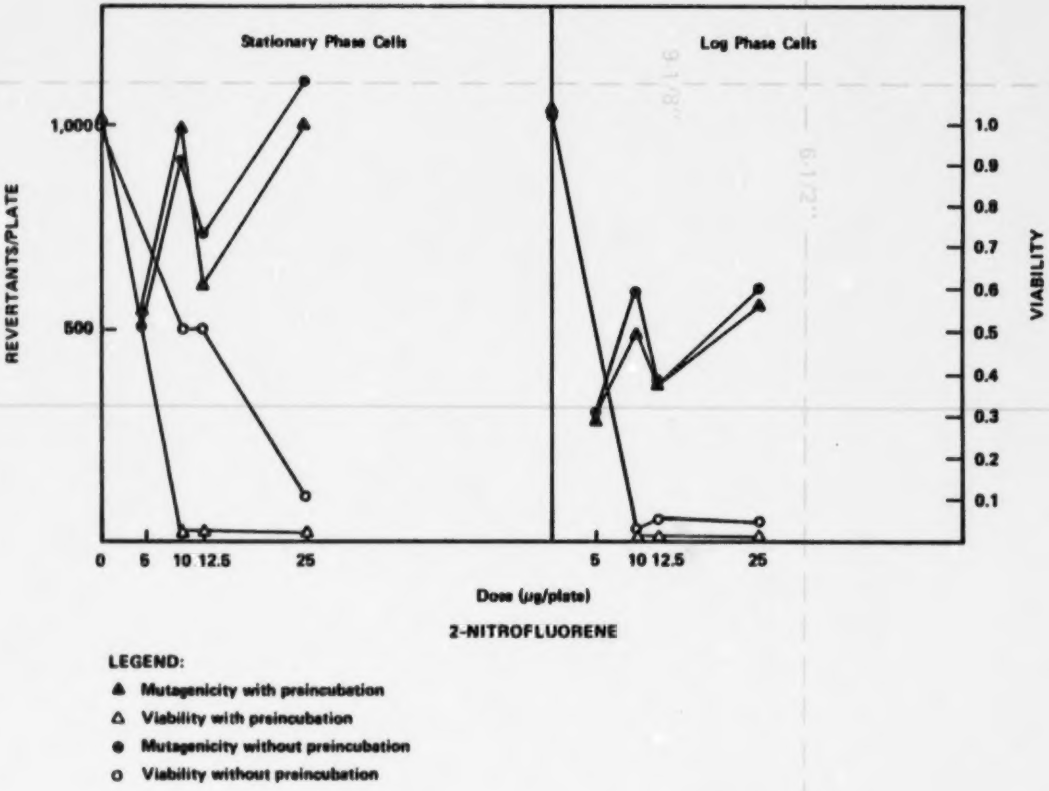

Figure 14. Mutagenicity and toxicity of 2-nitrofluorene with log- and stationary-phase cells of *Salmonella* strain TA98 (with and without preincubation).

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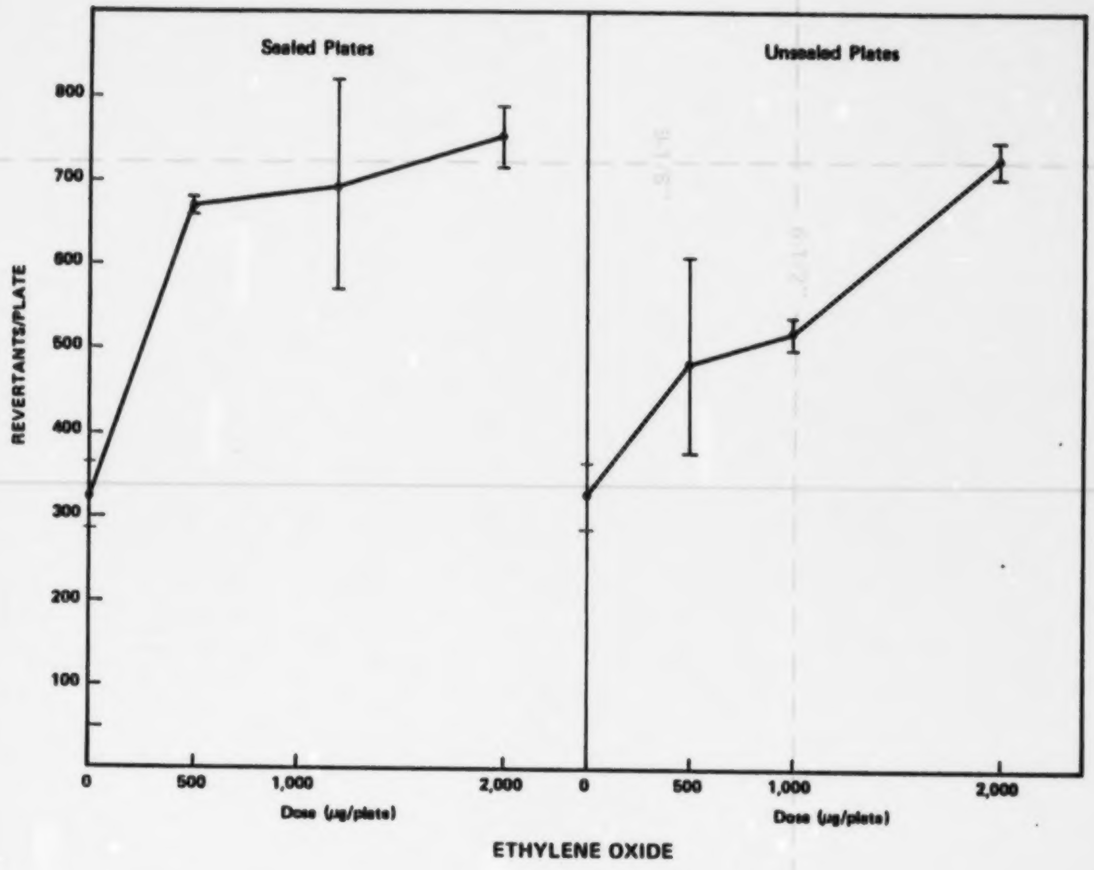

Figure 15. Effect of sealing plates with tape on the mutagenicity of ethylene oxide after preincubation with Salmonella strain TA98.

The above modifications to the preincubation technique of Yahagi et al. (1975) were included in all experiments with pure vapor-phase organic mutagens (see Results, Section 5). This modified protocol for the preincubation technique is contained in Table 7.

Verification of the chemical and biological stability of short-chain vapor-phase organic compounds after cryogenic freezing and storage at -80°C was performed to establish the effect of this procedure on vapor-phase compounds. The effect of thermal desorption on the chemical stability vapor-phase organic compounds had previously been verified (Pellizzari, 1977). Ethylene oxide, propylene oxide, and butylene oxide, all known volatile mutagens, were prepared in a glycerol/H_2O/95% ethanol solvent (2:2:1 v/v ratio) at concentrations of 3,000, 2,000, and 1,000 μg/plate. Duplicate samples were prepared; one sample was immediately analyzed by gas chromotagraphy (GC) and the other sample was frozen at -80°C for six days and retested by GC. The percentage recovery for each compound as determined by gas chromatography is plotted in Figure 16. Recovery of propylene oxide and butylene oxide was approximately 90% after one week of storage for the three sample concentrations tested. The recovery of ethylene oxide decreased to 65% of control values for the three concentrations tested after one week of storage. This experiment demonstrated that compounds for the biological validation of the preincubation technique should be tested immediately after preparation. However, this experiment also suggested that vapor-phase organic model compounds (and by implication field samples) could be stored for a minimum of one week with no appreciable loss of chemical activity.

A similar experiment was performed to evaluate the biological stability of propylene oxide after 20 days of storage at -80°C. Propylene oxide was prepared in glycerol/water/95% ethanol in a 2:2:1 v/v ratio, and tested with Salmonella strain TA100 in the standard pour plate assay (with log- and stationary-phase cells) and in the preincubation technique (with log-phase cells). Results are plotted in Figure 17. Similar to the chemical evaluation experiment discussed above, the freshly prepared sample of propylene oxide was more biologically active. However, even after 20 days of storage at -80°C, propylene oxide retained approximately 80% of the original mutagenic activity. The preincubation technique with log-phase cells was the most sensitive technique. Dose response curves for both freshly prepared and stored samples were essentially identical, except that the stored sample was approximately 20% less active than the fresh sample. These experiments suggested that the chemical and biological stability of vapor-phase organic compounds remained stable, even when stored at -80°C for three weeks. Consequently, the degree of hydrolysis and/or the loss of epoxides from frozen samples was minimal, as determined by these chemical and biological methodologies. These experiments further demonstrated that vapor-phase organic field samples would retain their biological and chemical activity for 1-3 weeks after sampling, when the vapor samples were immediately stored at -80°C.

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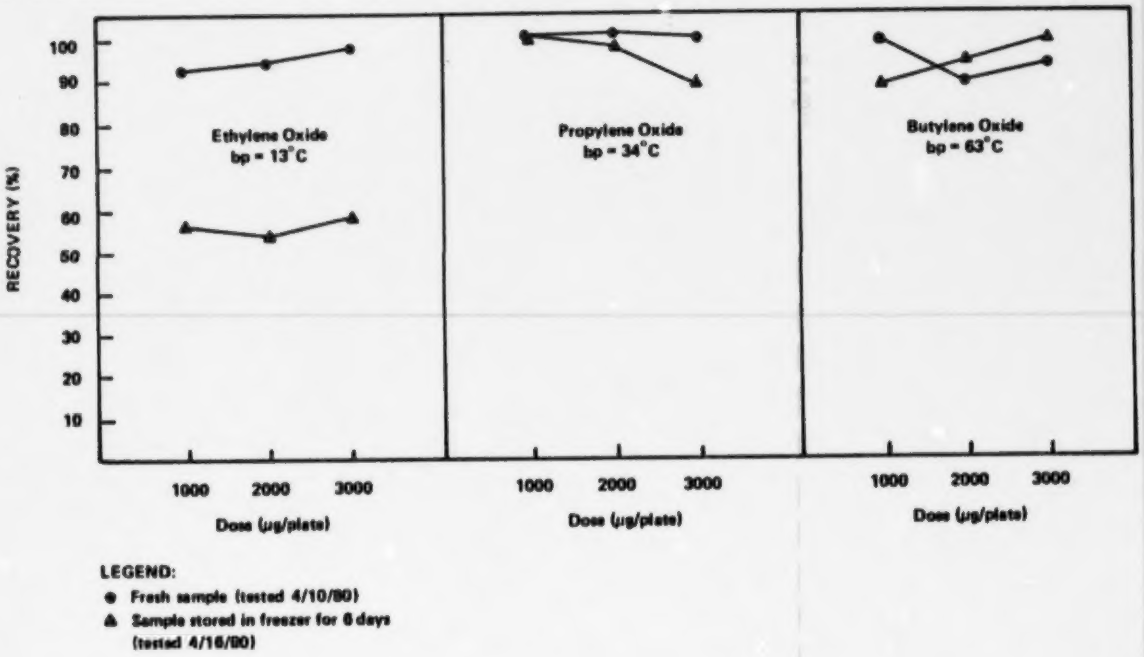

Figure 16. Recovery of ethylene, propylene and butylene oxides after storage at -80°C for six days, as measured by gas chromatography.

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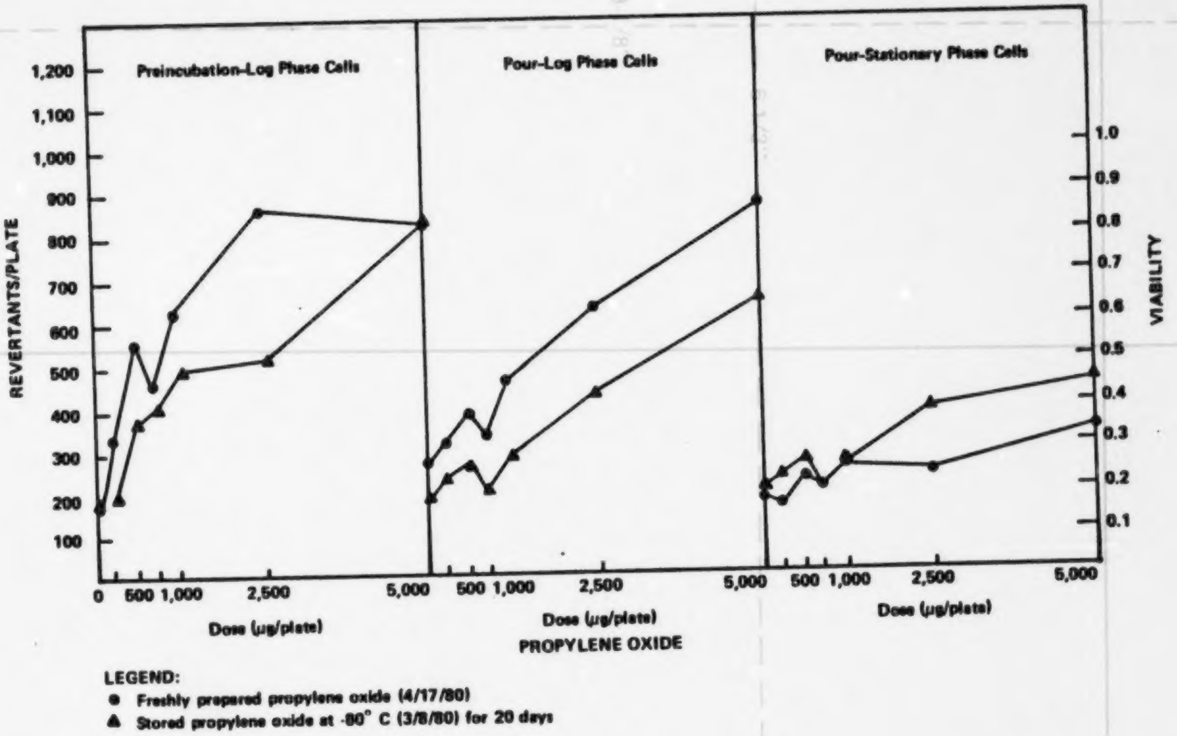

Figure 17. Mutagenicity of propylene oxide upon storage at ~80°C for 20 days, as measured by pour and preincubation techniques with Salmonella strain TA100.

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SECTION 5

RESULTS

FRACTIONATION AND MUTAGENICITY TESTING OF PARTICULATE ORGANIC MATTER (POM) IN AMBIENT AIR FROM SEVEN GEOGRAPHICAL LOCATIONS

Ambient air particulate samples were collected from seven geographical locations during the three year tenure of this contract (May 1977-May 1980). These samples were collected to aid in the development of collection, chemical and bioassay techniques and therefore were not intended to be utilized in health risk assessment. Explicit methodologies for all testing procedures are contained in Section 4 and in the Appendices (A, B, and C) and are briefly outlined below.

Particulate samples were collected with the MAVS and were fractionated by size into three classes: 3.5-20.0 µm, 1.7-3.5 µm, and <1.7 µm (average mean-diameter of the particles). After collection, the organic matter from these particles was solvent extracted in cyclohexane under sonication; this crude organic extract was then fractionated into six chemical-classes with a validated chemical fractionation scheme. The final six chemical-classes were: organic acids, organic bases, polar neutrals (PN), nonpolar neutrals (NPN), polynuclear aromatic hydrocarbons (PNA), and cyclohexane insolubles (INT). After fractionation, these individual chemical-classes and total crude extracts (when sample size permitted) were tested for mutagenic potential with the Ames/*Salmonella* mammalian microsome mutagenicity test. A mutagenic response in the Ames/*Salmonella* plate incorporation assay was considered positive if the increase in revertant colonies observed was twice the spontaneous reversion rate, and the increase in colonies observed increased with the dose level. However, since sample size was limited in this study, only two doses of each fraction were usually tested. Therefore, the second criteria for a positive mutagenic response was not met. Samples were therefore considered potentially mutagenic if one dose resulted in an increase in colonies that was twice the background rate. The criteria for a positive response in the agar well and spot test techniques were largely visual and therefore qualitative, and involved a significant increase in colonies in the vicinity of the sample. Chemical identification of signature mutagenic and/or carcinogen compounds was accomplished by GC/MS/Computer analysis.

The locations studied under this program were: South Charleston, West Virginia, and Baton Rouge, Louisiana in the first year of this contract, and Lake Charles, Louisiana; Beaumont, Texas; Houston, Texas; Upland, California, and Elizabeth, New Jersey in the second year of this

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contract. These sites were chosen because they were highly industrialized with major and diverse manufacturing, mining and petrochemical industries. Table 8 lists the cities and fractionation information for year 1 and Table 9 lists similar information for year 2. These summary tables highlight important facts concerning this testing; namely, that many chemical-classes contained less than 10 mg of total extractable organic material (which contained hundreds of individual chemicals) and that the chemical recovery from the crude extract generally exceeded 80%. Original particulate sample sizes were on the order of 2-4 g of particles; chemical extraction of organic material from the ambient air particles ranged from 10-20% on a weight/weight basis. Operating difficulties with the MAUS throughout the course of this program prevented the collection of samples of adequate quantity for complete bioassay, chemical fractionation and identification.

This limited sample size, which resulted from an initial small sample size and a low percentage of organic material extracted from the particles, precluded extensive dose response mutagenicity testing of samples from these locations with the Ames/Salmonella assay. Consequently, a modified bioassay testing procedure (the agar well diffusion technique) was developed to obtain the maximum amount of information per sample. A bioassay priority scheme was also developed, which outlined assay procedures, dose levels, activation/nonactivation, and strain sequences based on total sample size. Utilization of either the agar well technique or the standard pour plate technique for the Ames/Salmonella assay (Ames et al., 1975) depended on the amount of sample in each fraction. The agar well assay was utilized only in situations where the total amount of sample was less than 10 mg. When sample size was greater than 10 mg, the assay procedure employed was the standard plate incorporation technique (Ames et al., 1975b).

Mutagenicity testing under this program was secondary to development of biological and chemical testing procedures. Consequently, the preliminary testing of these seven locations is not considered adequate for quantitative mutagenic comparison. The data from these field sites was utilized for validation of both the biological and chemical testing procedures for future ambient air testing. However, even this very preliminary data indicated the need for more extensive and quantitative testing of ambient air from these and similar industrial sites.

The data obtained from these seven geographical locations follows; each site is discussed individually.

South Charleston, West Virginia (#1) Ambient Air Particulate Sample

This first air particulate sample tested under this program was collected in the Kanawha Valley, near southwest Charleston, West Virginia on August 22, 1977 by EPA personnel (Messrs. Richard Burton, William Bernard, and Randy Baxter of HERL/RTP). This site was chosen due to the high density of chemical manufacturing and rubber processing plants in this area. The first particulate sample tested was Sample I from South Charleston, West Virginia. The sample (1.0 g) consisted of only <1.7 μm

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TABLE 8. LOCATIONS AND FRACTIONATION INFORMATION FOR PARTICULATE SAMPLES COLLECTED DURING YEAR 1 (MAY 1977 - MAY 1978)

Particulate sample	Total particulate weight (gm)	Total organics extracted (mg)	Amount of organics in each fraction (mg)					% Recovery
			Acid	Base	Non-polar neutrals	PNA	Polar neutrals	
South Charleston, WV (#1)[a]	1.00	136.7	12.5	2.4	16.7	5.5	10.0	100.0
South Charleston, WV (#2)[a]	4.28	207.6	47.8	8.2	61.3	11.5	43.8	83.1
Baton Rouge, LA (<1.7 μm)	1.19	192.6	5.0	6.2	12.4	3.2	155.5	96.8
Baton Rouge, LA (>1.7 μm)	1.34	175.8	8.3	4.9	53.0	18.9	81.9	95.5

[a] Fractionated with first fraction scheme; all other samples were fractionated with second fraction scheme, which yield more sample in each chemical-class.

TABLE 9. LOCATIONS AND FRACTIONATION INFORMATION FOR PARTICULATE SAMPLES COLLECTED DURING YEAR 2 (MAY 1978 - AUGUST 1979)

Particulate sample	Total particulate weight (gm)	Total organics extracted (mg)	Amount of organics in each fraction (mg)					% Recovery
			Acid	Base	Non-polar neutrals	PNA	Polar neutrals	
Upland, CA (<1.7 µm)	0.67	32.7	2.9	1.0	6.5	1.6	15.8	85.0
Upland, CA (1.7-3.5 µm)	0.29	17.5	1.8	0.6	4.7	2.0	6.3	88.0
Upland, CA (>3.5 µm)	0.64	35.9	1.1	0.7	9.4	2.5	16.0	82.7
Houston, TX (<1.7 µm)	0.34	19.3	1.0	0.6	4.0	3.2	9.9	96.9
Houston, TX (>1.7 µm)	0.79	86.4	0.8	1.2	3.2	2.4	33.2	47.2
Lake Charles, LA #1 (>1.7 µm)	0.75	24.6	7.2	0.1	6.1	2.1	3.7	78.0
Lake Charles, LA #2 (>1.7 µm)	0.11	11.9	1.7	0.1	3.9	2.3	2.4	87.4
Elizabeth, NJ (>1.7 µm)	1.35	180.1	8.5	0.5	86.2	11.1	34.6	78.2
Elizabeth, NJ (1.7-3.5 µm)	1.30	119.7	10.4	0.4	47.6	9.6	26.1	78.6
Elizabeth, NJ (>3.5 µm)	1.44	99.4	5.5	0.2	52.6	6.0	13.4	78.2
Lake Charles, LA #2 (<1.7 µm)	2.62	401.2	170.2	16.3	49.2	7.1	152.6	98.6
Lake Charles, LA #2 (1.7-3.5 µm)	0.49	15.2	1.9	4.9	4.9	1.5	1.3	88.8
Lake Charles, LA #2 (>3.5 µm)	0.55	26.8	16.2	3.1	5.3	1.0	0.8	98.5
Beaumont, TX (<1.7 µm)	0.29	150.6	15.5	6.3	50.3	8.5	6.7	58.0
Beaumont, TX (1.7-3.5 µm)	0.88	30.7	6.8	0.6	12.2	2.2	8.9	100.0
Beaumont, TX (>3.5 µm)	1.70	42.1	10.4	0.4	21.3	4.8	3.8	96.7
Houston, TX #2 (<1.7 µm)	3.04	110.2	20.4	2.8	20.9	12.2	38.0	85.6
Houston, TX #2 (1.7-3.5 µm)	ND	ND	ND	ND	ND	ND	ND	ND
Houston, TX #2 (>3.5 µm)	ND	ND	ND	ND	ND	ND	ND	ND

ND = Not detected.

particles, and was subjected to chemical fractionation and analysis by the initial chemical fractionation scheme (see Figure 3). Approximately 14% (by weight) of the crude particulate sample consisted of organic extractables (Table 10). The fractions were subjected to further analysis with GC/MS/Computer analysis. Mutagenic signature compounds identified in the various fractions are listed in Table 45.

Upon fractionation of this first sample (1.0 g) from the Kanawha Valley, it immediately became apparent that the amount of material in some chemical-class fractions would be too small and would place severe limitations on the number of bioassay tests which could be conducted. Depending on sample size, a decision had to be made regarding number of strains, number of replicates, type of test (spot or plate incorporation), and number of doses tested (single dose or dose response curve). Consequently, in order to investigate the above experimental testing parameters, each fraction from this initial particulate sample from Kanawha Valley was diluted with DMSO to a final volume of 3.0 ml. This was performed to provide sufficient volume for tests with all five strains. As a consequence, the dose per plate varied from fraction to fraction. These tests were conducted with incorporation the histidine and biotin in the base layer (Frantz and Malling, 1975) rather than in the top layer (Ames et al., 1975). Tables 11 to 13 summarize results of spot and plate incorporation testing of the chemical-class fractions. Results are also shown for the amount of material available for testing; most fractions contained less than 15 mg. Positive results with the spot test technique were obtained with most Level 1 fractions (Table 11), but interpretation was difficult since the doses precluded adequate dose response testing. In a few cases, dose response studies with the pour plate technique were attempted. The point that must be stressed is that very small sample size necessitated limited testing and a negative result in this situation cannot be interpreted as having much significance. With a small sample size, there is no way to determine a dose response curve (Katz, 1978). In addition, if only spot tests were performed, there is no method to determine if there were compounds in the fraction which could only be properly evaluated with plate incorporation tests. For example, benzo(a)pyrene will not migrate in the water based agar layer, and therefore was only detected as a mutagen in the pour plate test (see Appendix C). Nonetheless, mutagenic activity with the spot test was detected in Level 1 polar (T3) and nonpolar (T1) organic fractions with strain TA98, TA100, and TA1538 (Table 11). Activity did not require S9 metabolic activation; therefore, these were direct-acting mutagens. Pour plate incorporation tests were negative, but this may be due to a dilution effect. Results from Level 2 (Table 12) suggested positive mutagenic results in the polar base (T5b) and polar acid (T7b) fractions. Activity was again direct-acting (no S9 requirement) which correlated with the presence of a direct-acting mutagen (2-nitro-4,6-dichlorophenol) in the base fraction (Table 45). It was noted that when adequate sample was available with these two fractions (T5b and T7b fractions) activity was detected; no activity was detected with a smaller sample size (T5a and T7a fractions). Mutagenic activity in the polar base fraction (T5b) was confirmed in a preliminary pour plate incorporation test with TA98 at 1440 µg/plate. The polar acid fraction (T7b) demonstrated direct-acting

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TABLE 10. EXTRACTION/FRACTIONATION RESULTS FROM SOUTH CHARLESTON, WEST VIRGINIA (I) AIR PARTICLES (<1.7 μm)[a]

Particulate (g) ~1.0	Wgt. Extracted (mg) 136.7	% Extracted 13.67
Fraction	**Wgt. (mg)**	**% of Total Extracted**
Nonpolar (T1)	75.4	55.0
Polar (T3)	20.3	15.0
Inorganic (T2)	863.3	-
Polar Bases (T5)	1.8	1.0
Polar Acids (T7)	8.5	6.0
Polar Neutrals (T6)	10.0	7.0
Nonpolar Bases (T9)	0.6	<1.0
Nonpolar Acids (T11)	4.0	3.0
Nonpolar Neutrals (N) (T12)	16.7	12.0
Nonpolar Nuetrals (P) (T13)	5.5	4.0
TOTAL RECOVERY	142.8 mg (organic classes)	104.0 %

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[a] Fractionated with initial 13 chemical-class fractionation scheme (Figure 3).

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TABLE 11. MUTAGENIC TESTING OF FRACTIONS FROM LEVEL 1 FRACTIONATION OF PARTICLES FROM SAMPLE 1 FROM SOUTH CHARLESTON, WEST VIRGINIA

Fraction	Description Chemical Type + Total Sample Amount	Dose µg/plate	Strain	Results, Av. NO. Colonies/Plate NO S-9	Results, Av. NO. Colonies/Plate Induced S-9	Mutagenic Ratio
A. SPOT TESTS						
T_1	Non-Polar Organics (23.6 mg)	790	98	19[a]	TNTC[a,b]	
		"	100	0	0	
		"	1535	2	2	
		"	1537	5	7	
		"	1538	3	6	
T_2	-- NOT TESTED --					
T_3	Polar Organics (~0.78 mg)	26	98	0	0	
		"	100	0	0	
		"	1535	2	3	
		"	1537	6	8	
		"	1538	2	2	
T_3	Polar Organics (7.5 mg)	250	98	12[a]	6	
		"	100	0	0	
		"	1535	5	0	
		"	1537	2	3	
		"	1538	4	0	
T_{3a}	Polar Organics (12.0 mg) (Portion insoluble in methanol)	400	98	0	0	
		"	100	TNTC[a]	0	
		"	1535	2	2	
		"	1537	5	7	
		"	1538	1	1	
T_3	Polar Organics (12.9 mg)	430	98	25[a]	15	
		"	100	0	0	
		"	1535	3	6	
		"	1537	9	6	
		"	1538	28[a]	16[a]	
B. POUR PLATE TESTS OF POSITIVE FRACTIONS						
T_1	Non-polar Organics (23.6 mg)	790	98	89[a]		1.9
T_3	Polar Organics (7.5)	250	98	63		1.3
T_{3a}	Polar Organics (12.0 mg) (portion insoluble in methanol)	400	1535 100	43 46		1.3 0.9
T_3	Polar Organics (12.9 mg)	430	98	106[a]		2.3

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[a] Positive results.

[b] TNTC = Too numerous to count (>200 colonies).

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TABLE 12. MUTAGENESIS TESTING OF FRACTIONS FROM LEVEL 2 FRACTIONATION (POLAR ORGANICS) OF PARTICLES FROM SAMPLE 1 FROM SOUTH CHARLESTON, WEST VIRGINIA

Fraction	Description Chemical Type + Total Sample Amount	Dose µg/plate	Strain	Results, Av. No. Colonies/Plate NO S-9	Results, Av. No. Colonies/Plate Induced S-9	Mutagenic Ratio
A. SPOT TESTS						
T_4	Polar acids and neutrals (1.0 mg)	33	98	0	0	
			100	0	0	
			1535	7	2	
			1537	5	4	
			1538	3	2	
T_{5a}^{c}	Polar bases (0.9 mg)	30	98	0	0	
			100	0	0	
			1535	1	1	
			1537	7	3	
			1538	1	1	
T_{5b}	Polar bases (43.2 mg)	1440	98	35^a	28^a	
			100	0	0	
			1535	2	5	
			1537	16^a	7	
			1538	34^a	5	
T_6	Polar neutrals (2.1 mg)	70	98	5	0	
			100	0	0	
			1535	3	2	
			1537	4	5	
			1538	2	3	
T_{7a}	Polar acids (1.5 mg)	50	98	0	0	
			100	0	0	
			1535	2	3	
			1537	5	6	
			1538	3	2	
T_{7b}	Polar acids (9.0 mg)	300	98	TNTC^a	TNTC^a	
			100	TNTC^a	TNTC^a	
			1535	2	TNTC^a	
			1537	TNTC^a	TNTC^a	
			1538	TNTC^a	TNTC^a	
B. POUR PLATE TESTS OF POSITIVE FRACTIONS						
T_{5b}	Polar bases (43.2 mg)	1440	98	154^a		3.3^a
T_{7b}	Polar acids (9.0 mg)	300	1535	36		1.0
			100	47		0.7
			98	70		1.7

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[a] Positive results.

[b] TNTC = Too numerous to count.

[c] 5a and 5b, and 7a and 7b are replicate samples, differing only in amount of starting material.

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TABLE 13. MUTAGENESIS TESTING OF FRACTIONS FROM LEVEL 3 FRACTIONATION (NONPOLAR ORGANICS) OF PARTICLES FROM SAMPLE 1 FROM SOUTH CHARLESTON, WEST VIRGINIA

Fraction	Description Chemical Type + Total Sample Amount	Dose µg/plate	Strain	Results, Av. No. Colonies/Plate		Mutagenic Ratio
				NO S-9	Induced S-9	
A. SPOT TESTS						
T_8	Nonpolar acids and neutrals (in DMSO)		98	0	0	
			100	0	0	
			1535	2	TNTC[a]	
			1537	9	6	
			1538	0	0	
T_8	Nonpolar acids and neutrals (0.2 mg)	6	98	0	0	
			100	0	0	
			1535	3	3	
			1537	3	6	
			1538	1	2	
T_9	Nonpolar bases (0.6 mg)	20	98	0	0	
			100	0	0	
			1535	3	2	
			1537	3	4	
			1538	0	0	
T_{10}	Nonpolar neutrals (0.4 mg)	13	98	0	0	
			100	0	0	
			1535	3	5	
			1537	9	4	
			1538	3	2	
T_{11}	Nonpolar acids (17.4 mg)	580	98	0	0	
			100	0	0	
			1535	3	3	
			1537	4	4	
			1538	0	1	
T_{12}	Parafins (1.7 mg)	56	98	0	0	
			100	0	6	
			1535	4	4	
			1537	5	5	
			1538	2	1	
T_{13}	Aromatics (1.3 mg)	43	98	0	0	
			100	0	TNTC[a]	
			1535	3	2	
			1537	4	6	
			1538	2	4	
B. POUR PLATE TESTS OF POSITIVE FRACTIONS						
T_8	Nonpolar acids and neutrals (in DMSO)	--	1535		32	1.0
			100		47	0.9
T_{13}	Aromatics (1.3 mg)	43	1535		18	0.7
			100		35	0.5

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[a] TNTC = Too numerous to count, positive results

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mutagenic activity with every tester strain except TA1535; TA1535 was active with S9 addition. Results from Level 3 (nonpolar organics), shown on Table 13, demonstrated activity in the nonpolar acids and neutrals (T8) and in the aromatic fractions (T13) with S9 metabolic activation. Active strains were TA1535 and TA100 respectively. Consequently, qualitative mutagenic activity was demonstrated with all five Salmonella tester strains, and both direct-acting mutagens and promutagens were present.

As a result of the testing with Sample I from the Kanawha Valley, West Virginia, it was necessary to reconsider the basic approach to integrated chemical and microbiological testing of air samples. In order to resolve the problems of limited sample size, the following options were considered in further testing:

(1) development of a tighter fractionation scheme with less fractions;

(2) modification of the Salmonella/microsome mutagenicity assay to give more information per unit of sample;

(3) prioritization of parameters (number of strains, activation requirements, dose response, replicates) to be employed when sample size does not permit complete testing.

A priority scheme based on the above considerations was therefore developed to optimize testing capabilities (see Development of Methods - Section 4; Table 4).

South Charleston, West Virginia (#2) Ambient Air Particulate Sample

The second particulate sample (4.28 g) was also collected from the Kanawha Valley on September 22, 1977 and was fractionated with the initial fractionation scheme (see Figure 3). Again, only the <1.7 μm fraction was bioassayed. Chemical components identified in this sample are shown in Table 45 and the weight of each fraction is shown in Table 14. The majority of the organic extractables (80%) was categorized as "nonpolar" by cyclohexane and ultrasonic treatment.

In these tests the decision was made to test the sample as thoroughly as possible with one strain, rather than to test the sample with all five strains. In these tests, the histidine and biotin were incorporated into the agar overlay as recommended by Ames et al. (1975b). Results with strain TA98 are shown in Tables 15 to 17. This series of tests enabled comparison of the well, spot, and pour plate incorporation techniques with an actual sample. The three techniques gave comparable results with the exception of the T1 fraction which showed no activity in the well and spot test, but did demonstrate a positive mutagenic response (MR = 4.0) in plate incorporation tests with the addition of S9. The polar base fraction (T5) also demonstrated a mutagenic response in the pour plate test (MR = 2.1 with S9 addition) but no activity was evident with the well test. T5 was not tested in the spot test due to

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TABLE 14. EXTRACTION/FRACTIONATION RESULTS FROM SOUTH CHARLESTON, WEST VIRGINIA (II) AIR PARTICLES (<1.7 μm)[a]

Particulate (g) 4.28	Wgt. Extracted (mg) 207.6	% Extracted 4.8
Fraction	Wgt. (mg)	% of Total Extract
Non-polar (T1)	107.3	51.7
Polar (T3)	100.3	48.3
Inorganic (T2)	3876	-
Polar Bases (T5)	5.4	2.6
Polar Acids (T7)	32.3	15.6
Polar Neutrals (T6)	43.8	21.1
Non-polar Bases (T9)	2.8	1.3
Non-polar Acids (T11)	15.5	7.5
Non-polar Neutrals (N) (T12)	61.3	29.5
Non-polar Neutrals (P) (T13)	11.5	5.5
	172.6 mg Total Recovery (organic classes)	83.1% Total Recovery

[a] Fractionated with initial 13 chemical-class fractionation scheme (Figure 3).

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TABLE 15. MUTAGENESIS TESTING OF CHEMICAL-CLASS FRACTIONS FROM AMBIENT AIR PARTICLES FROM SAMPLE II FROM SOUTH CHARLESTON, WEST VIRGINIA (<1.7 μm) WITH SALMONELLA STRAIN TA98 - WELL TEST

			Well Test (5 Well Configuration) Avg. No. of Colonies Around Appropriate Wells, 2 Plates		
Fraction	Description	Available Sample, mg	Positive Control Well Anthramine, 100 μg	Sample Well 100 μg	Sample Well 500 μg
T 1	Non-polar Organics	14.8	23	0	3
T 3	Polar Organics	16.1	27	23[a]	36[a]
T 5	Polar Bases	2.1	26	2	1
T 6	Polar Neutrals	12.7	26	32[a]	14[a]
T 7	Polar Acids	5.6	31	31[a]	37[a]
T 9	Non-polar Bases	1.3	18	0	0
T 11	Non-polar Acids	5.3	25	0	1
T 12	Paraffins	15.3	27	0	0
T 13	Non-polar Neutrals	3.6	31	0	0
Solvent Control (DMSO)	100 μl	0	0	0	0

[a] Positive mutagenic response: number of colonies in immediate vicinity of sample well.

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TABLE 16. MUTAGENESIS TESTING OF CHEMICAL-CLASS FRACTIONS FROM AMBIENT AIR PARTICLES FROM SAMPLE II FROM SOUTH CHARLESTON, WEST VIRGINIA (<1.7 μm) WITH SALMONELLA STRAIN TA98 - SPOT TEST

			Spot Tests, 500 μg/plate Avg. No. of Colonies, 2 Plates	
Fraction	Description	Available Sample, mg	+S9 Microsomes	-S9 Microsomes
T 1	Non-polar Organics	14.8	159	155
T 3	Polar Organics	16.1	278[a]	206[a]
T 5	Polar Bases	2.1	-	-
T 6	Polar Neutrals	12.7	217[a]	176[a]
T 7	Polar Acids	5.6	252[a]	223[a]
T 9	Non-polar Bases	1.3	-	-
T 11	Non-polar Acids	5.3	147	130
T 12	Paraffins	15.3	136	121
T 13	Non-polar Neutrals	3.6	167	145
Solvent Control (DMSO)	100 μl	0	109[b]	88[b]

[a] Positive mutagenic response.

[b] High spontaneous background rate.

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TABLE 17. MUTAGENESIS TESTING OF CHEMICAL-CLASS FRACTIONS FROM AMBIENT AIR PARTICLES FROM SAMPLE II FROM SOUTH CHARLESTON, WEST VIRGINIA (<1.7 μm) WITH SALMONELLA STRAIN TA98 - PLATE INCORPORATION TEST

			Plate Incorporation Tests 500 μg/plate Avg. No. Coloines/2 Plates		Mutagenic Ratio[b]	
Fraction	Description	Available Sample, mg	+S9 Microsomes	-S9 Microsomes	+S9 Microsomes	-S9 Microsomes
T 1	Non-polar Organics	14.8	123[a]	43[a]	4.0[a]	2.0[a]
T 3	Polar Organics	16.1	250[a]	161[a]	8.1[a]	7.3[a]
T 5	Polar Bases	2.1	65[a]	42	2.1[a]	1.9
T 6	Polar Neutrals	12.7	172[a]	66[a]	5.5[a]	3.0[a]
T 7	Polar Acids	5.6	160[a]	106[a]	5.2[a]	4.8[a]
T 9	Non-polar Bases	1.3	27	16	0.9	0.7
T 11	Non-polar Acids	5.3	33	25	1.1	1.1
T 12	Paraffins	15.3	55	35	1.8	1.6
T 13	Non-polar Neutrals	3.6	-	-	-	-
Solvent Control (DMSO)	100 μl	0	31	22	1.0	1.0

[a] Positive mutagenic response.

[b] Mutagenic ratio = No. of colonies with sample/no. of colonies without sample but with solvent.

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lack of sample. Since the T1 fraction was derived from Level 1 fractionation and since the initial scheme demonstrated spillover of components from one fraction to another fraction, it was possible that the T1 fraction contained some compound which was not detectable except in the pour plate incorporation test. Direct mutagenic activity was detected in fractions T3 (polar organics), T6 (polar neutrals), and T7 (polar bases) with all three techniques. Activity did increase slightly with S9 addition. These results suggested that the agar well and spot test techniques would detect similar mutagenic activity in ambient air particles. However, the pour plate technique was the assay-of-choice, especially when weak mutagens were present [*e.g.*, T1 (nonpolar organics) and T5 (polar bases), whose mutagenis ratios were 4.0 and 2.1 respectively]. Chemical identification of signature mutagenic/carcinogenic compounds in these fractions (Table 45) correlated with the observed mutagenic activity. The highest mutagenic potential was observed in the polar organic (T3), polar neutrals (T6) and polar acid (T7) fractions; the greatest number of mutagenic/carcinogenic compounds was identified in the polar neutral fractions.

Baton Rouge, Louisiana Ambient Air Particulate Sample

The third sample was obtained from Baton Rouge, LA., on November 11, 1977. During collection, the particulates were subdivided into two samples by size. The <1.7 μm size particles had a total size of 1.19 g, and the >1.7 μm size particles had a total sample size of 1.34 g. This was the first sample tested which utilized the final chemical fractionation scheme (Figure 2) together with the agar diffusion well test (Appendix C) and the bioassay priority scheme (Table 4). Organic compounds identified in these samples are shown in Table 45 with the weights of each fraction in Tables 18 and 19. The majority of organic extractable materials were fractionated into the polar neutrals. Initial results of mutagenesis testing are in Table 20. The approach to mutagenicity testing of this sample was based on the studies with previous samples and with known compounds. Since PNAs do not diffuse readily into agar, the PNA fractions were tested directly with the plate incorporation technique with TA98 and with Aroclor-induced S9 from CD_1 male rat liver. All other fractions were initially tested in the agar well test. Initial mutagenicity results are in Table 20, and these results demonstrated significant mutagenic activity in the <1.7 μm particulate sample in the acid and PNA fractions with *Salmonella* strains TA1537 and TA98. This activity was direct-acting, but S9 addition did increase activity both for the acid and PNA fraction (see Table 21). The >1.7 μm particulate sample demonstrated activity in the PNA fraction with S9 addition (Table 20) and *Salmonella* strains TA1537, TA1538, and TA98 at 500 μg/plate. The basic fraction (>1.7 μm) was active in the well test at 500 μg with strain TA1537 (Table 20). These results suggested greater mutagenic activity in the smaller (<1.7 μm) particulate sample for the PNA and acid fractions. Mutagenic activity was direct-acting. Results with TA1535 are not interpretable due to control values that were unusually high. Examination of chemical content of these fractions (Table 45) indicated the presence of phenol in the acid fraction of the <1.7 μm particulate sample; phenol is a known carcinogen (U. S. DHEW, 1977).

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TABLE 18. EXTRACTION/FRACTIONATION RESULTS OF BATON ROUGE, LOUISIANA AIR PARTICULATE SAMPLE (<1.7 μm)[a]

Particulate (g) 1.19	Wgt. Extracted (mg) 192.6	% Extracted 15.3
Fraction	**Wgt. (mg)**	**% of Total Extract**
Acids	5.0	2.6
Bases	6.2	3.2
Non-Polar Neutrals	12.4	6.4
PNA's	3.2	1.7
Polar Neutrals	155.5	80.7
Insolubles	0.3	0.2
	182.6 mg Total Recovery	94.8% Total Recovery

[a] This and all remaining samples were fractionated with the final six chemical-class fractionation scheme (Figure 2).

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TABLE 19. EXTRACTION/FRACTIONATION RESULTS OF BATON ROUGE, LOUISIANA AIR PARTICULATE SAMPLE (>1.7 μm)

Particulate (g)	Wgt. Extracted (mg)	% Extracted
1.34	175.8	13.1
Fraction	Wgt. (mg)	% of Total Extract
Acids	8.3	4.7
Bases	4.9	2.8
Non-Polar Neutrals	53.0	30.1
PNA's	18.9	10.8
Polar Neutrals	81.9	46.6
Insolubles	0.8	0.5
	167.8 mg Total Recovery	95.5% Total Recovery

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TABLE 20. INITIAL MUTAGENESIS TESTING OF PARTICULATE FRACTIONS FROM BATON ROUGE, LOUISIANA[a]

Fraction	Total Amt. of Sample	S-9 Required for Activity	Type Plate	Results Avg. No. of Colonies, Avg. of 2 Plates									
				TA 1535[c]		TA 1537		TA 1538		TA 98		TA 100	
				500 µg	100 µg	500 µg	100 µg	500 µg	100 µg	500 µg	100 µg	500µg	100 µg
Base < 1.7 µm	0.9 mg		Well	No sample		No sample		No sample		8	1	No sample	
Acid < 1.7 µm	3.0 mg	No	Well	0	0	79[b]	47[b]	No sample		111[b]	53[b]	No sample	
PNA < 1.7 µm	3.2 mg	No	Pour	167	Not tested	66[b]	Not tested	No sample		795[b]	Not tested	133	Not tested
Paraffins < 1.7 µm	8.0 mg		Well	0	0	0	0	0	0	0	0	0	3
Polar Neutrals < 1.7 µm	55.2 mg		Well	0	0	1	0	1	2	0	0	0	0
Base > 1.7 µm	4.9 mg	Yes	Well	0	0	26[b]	2	0	0	0	0	0	0
Acid > 1.7 µm	4.0 mg		Well	0	0	0	0	0	0	0	0	No sample	
PNA > 1.7 µm	10.9 mg	No	Pour	109	Not tested	113[b]	Not tested	236[b]	Not tested	461[b]	Not tested	149	Not tested
Paraffins > 1.7 µm	35.3 mg		Well	0	0	1	0	0	0	0	0	0	0
Polar Neutrals > 1.7 µm	72.9 mg		Well	0	0	6	3	8	4	3	3	0	0
Controls	0		Well	30	30	2	2	3	3	0	0	50	50
DMSO + S-9	0		Pour	136[c]		18		34		24		156	

[a] Well, and pour plate tests were performed as described in Materials and Methods (Section 4.1).

[b] A positive mutagenic response. PNA fraction activity was increased by the addition of the S9 microsomal preparation; activity was also detected in the absence of S9, however (Table 21).

[c] The number of spontaneous revertants with TA1535 was unusually high in the pour test because of the longer than usual incubation time.

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The mutagenically active PNA fractions for both particle sizes (<1.7 μm and >1.7 μm) contained the greatest number of known mutagenic/carcinogenic compounds. No mutagenic activity or mutagenic signature compounds was detected in either the polar neutral or nonpolar neutral fractions (<1.7 μm). The activity of the PN fraction in the >1.7 μm particles (Table 21) was probably due to the spillover of PNA type compounds (benzo(a)pyrene) in the PN fraction. This spillover can also be verified by the fact that the PN fraction (>1.7 μm) required metabolic activation for activity and mutagenic results were only demonstrated in the pour plate technique. The base fraction of the >1.7 μm sample was also mutagenically active (Table 20) and contained a number of known mutagenic compounds. Although the total number of mutagens/carcinogens identified was small, all identified mutagenic compounds in the base fraction were potent mutagens (Table 45).

A relatively large amount of the polar neutral fraction (>1.7 μm) was available, so pour plate dose response tests were conducted with TA98. Figure 18 shows the effects of varying both dose level and amount of S9 on the mutagenic response. Linear dose responses were obtained with 4 and 6 mg of S9 microsomal protein per plate. At 2 mg of S9 protein per plate, the mutagenic response was greater at lower concentrations of sample than at higher concentrations. This response was difficult to interpret; however, this type of response has been shown to occur with benzo(a)pyrene (Commoner et al., 1976 and Figure 19). Benzo(a)pyrene, a PNA compound, was a contaminant in this fraction (Table 45) and was probably responsible for this mutagenic activity. Figure 18 indicates that significant mutagenic activity (*i.e.*, mutagenic ratio greater than 3.0) in the polar neutral fraction required a maximum of 2 mg S9 protein/plate and a dose of 250 μg PN/plate. This dose response of the PN fraction agreed with other air particulate studies. Tokiwa et al. (1977) utilized chemical extraction and strain TA98 to demonstrate that a linear mutagenic response required from 100-400 μg of a methanol extract from an industrial particulate sample. However, weakly mutagenic air samples from residential sites required from 370-1970 μg/plate in order to achieve significant mutagenic activity. For this reason, 100 and 500 μg in the well test were chosen for initial testing with each strain (see Appendix C). Environmental testing may be therefore drastically different (in terms of total sample amounts required) as opposed to testing with pure compounds. Pure mutagenic compounds can be detected at 1 μg or less (McCann et al., 1975). However, for environmental samples, obtaining this amount of any single mutagenic compound may require many grams of total particulate sample. In addition, total extractable organic compounds accounted for only 5-10% of the total weight of the particulate samples, which further decreased the sample size. Results demonstrated that a minimum of 30-50 g of ambient air particulate sample was necessary for initial chemical and biological analysis. This amount of total sample would guarantee that each chemical-class fraction would contain a minimum of 50 mg of extractable organic extract, which was a minimal amount for initial dose responsive testing in the *Salmonella* assay with the plate incorporation technique.

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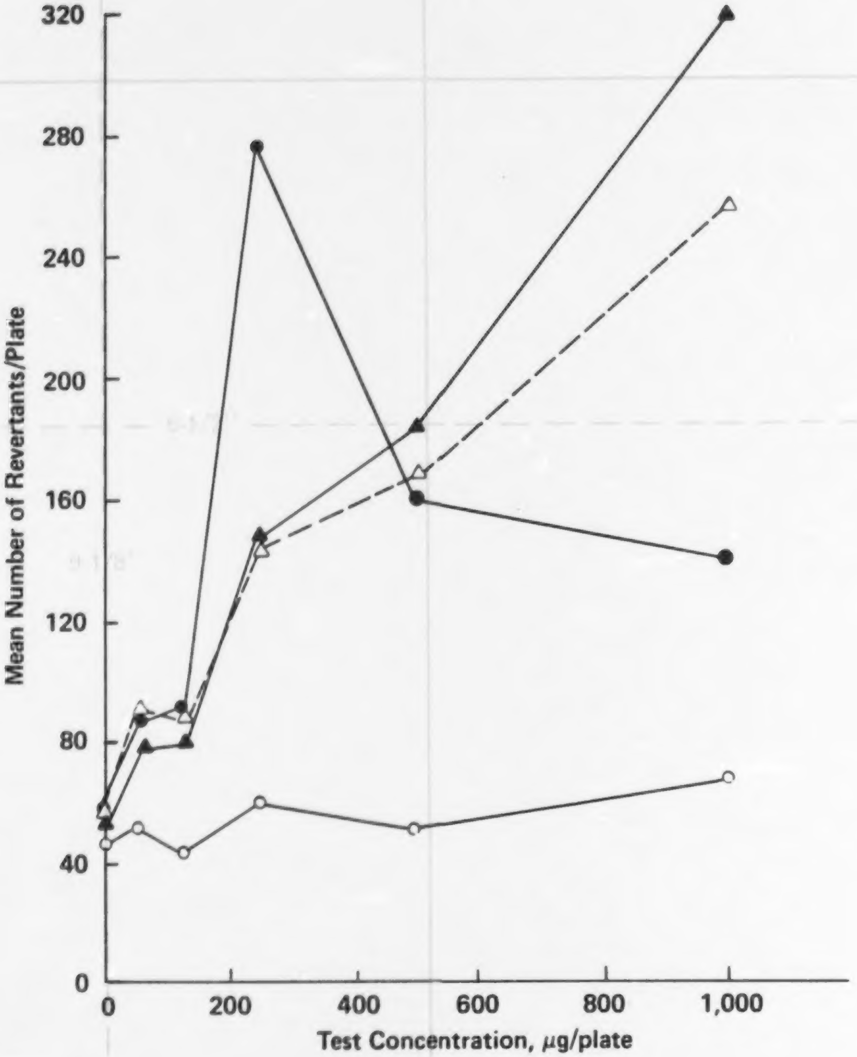

Figure 18. Dose response from Baton Rouge polar neutrals (>1.7 μm) with TA98 and the plate incorporation test. S-9 mg of protein per plate = ○ 0, ● 2, △ 4, ▲ 6.

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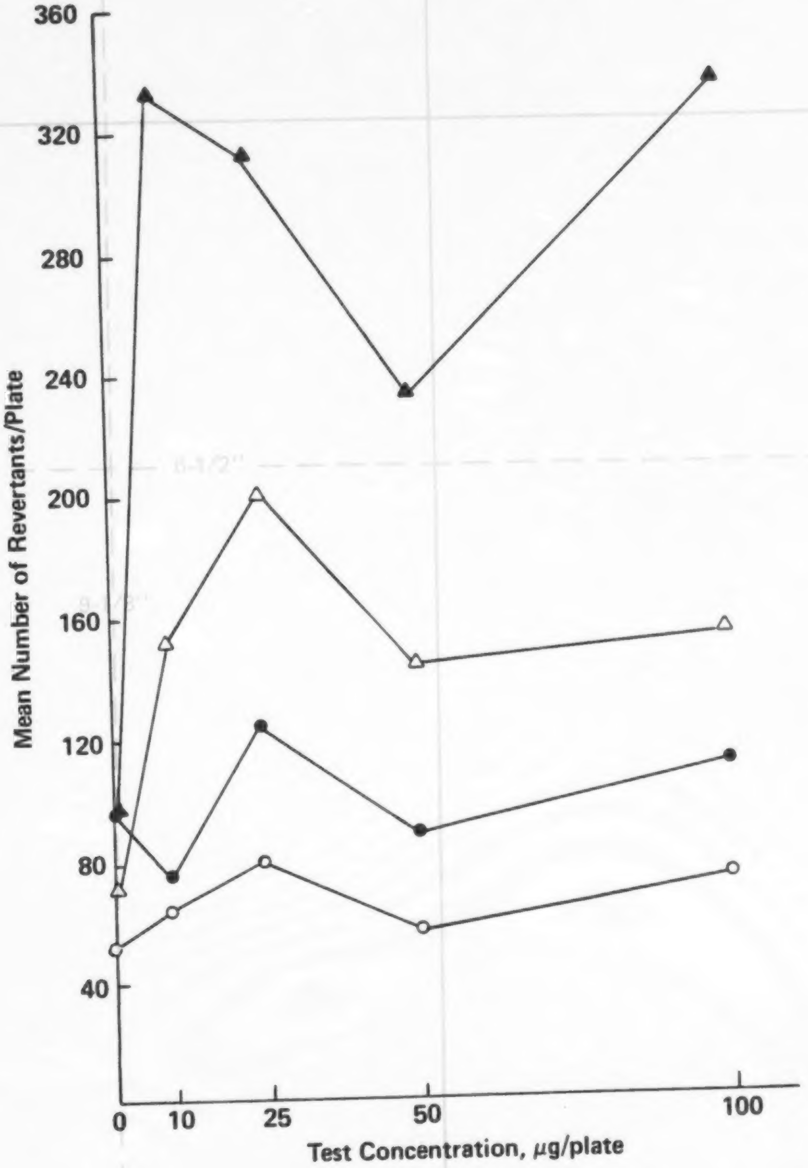

Figure 19. Dose response of benzo(a)pyrene with TA98 at 100, 50, 25, 10, and 1 μg/plate with the plate incorporation assay. S-9 mg of protein per plate = ○ 0.8, ● 1.5, △ 3.0, ▲ 6.0.

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An adequate amount of sample from Baton Rouge, Louisiana also allowed comparative testing of the well, spot, and pour plate techniques with an actual sample. Mutagenic and toxic ratios were determined with strain TA98. Results are in Table 21. No mutagenic activity was detected with any of the three techniques in the nonpolar netural (NPN) and polar neutrals (PN) fraction of the <1.7 μm particulate sample. This lack of mutagenic activity correlated with the absence of signature mutagens in these fractions (Table 45). In the >1.7 μm particulate sample, toxicity in the PNA fraction was evident (a viability ratio of 0.04), but was neutralized by the addition of S9 (Table 21). In addition, toxicity was also present in the polar neutral (PN) fraction without S9 addition. Mutagenicity of the PNA fraction was detected in the >1.7 μm fraction both with and without S9 addition in both the spot and pour plate tests. Mutagenic activity in the >1.7 μm size particulate was also detected in the PN fraction in the spot and pour plate tests, but not in the agar well test. The inability of the well test to detect these mutagens may be due to the spillover of PNAs into the PN fraction. PNAs are water-insoluble compounds and therefore would not be detected in the agar well test. Evidence that PNAs did spillover into the PN fraction of the >1.7 μm particulate sample was evidenced by the following facts: (1) the PN fraction required S9 addition for maximum expression; (2) a strongly positive mutagenic response was only obtained in the pour plate assay; and (3) chemical analysis of the polar neutral fraction demonstrated the presence of PNA-type compounds such as benzo(a)pyrene and benzfluoranthene (Table 45). No mutagenic activity was detected with any technique when the paraffins (NPN fraction) were tested with TA98. These results further suggested that the pour plate technique was the most sensitive method for detection of ambient air particulate mutagenicity, and that the agar well test and the spot test were useful when sample size was small.

Lake Charles, Louisiana Ambient Air Particulate Sample

Two air particulate samples were collected from Lake Charles, Louisiana; the first sample was collected in October 1978 and the second sample was collected on February 16-25, 1979. Both samples were collected at the Sheraton Chateau Charles Hotel, on the corner of RT 90W and Interstate 10. The primary industrial emissions from this area result from petroleum refinery operations and petrochemical, paint, and chemical manufacturing industries. Illegal industrial dumping sites were also a possible source of pollutant emissions.

The extraction and fractionation results for the two sampling trips to Lake Charles are summarized in Table 22. The first visit to Lake Charles in October 1978 generated 747 mg of <1.7 μm particles and 113.6 mg of >1.7 μm particles. Because of the small overall sample size collected on this first visit, the 1.7-3.5 μm and 3.5-20 μm size fractions were combined into one sample size (>1.7 μm) for organic extraction. During the second visit to Lake Charles in February 1979, 2618 mg of <1.7 μm, 488 mg of 1.7-3.5 μm, and 545 mg of >3.5 μm particulate sample was collected from 158,000 m^3 air sampled over 153.5 hr. The organic material from the particles was extracted as described in the Materials

TABLE 21. MUTAGENICITY AND TOXICITY TESTING OF BATON ROUGE AIR PARTICULATE FRACTIONS WITH TA98 IN THREE TECHNIQUES: WELL, SPOT, AND POUR PLATE TESTS[a]

	RESULTS															
	Well Tests (5 well configuration)			Spot Tests				Plate Incorporation Tests								
								Mutagenicity Tests, 500 μg				Toxicity Tests, 500 μg/plate				
	Avg. No. Colonies Around Appropriate Wells, 2 Plates			Avg. No. Colonies 2 Plates				Avg. No. Colonies 2 Plates		Mutagenic Ratio		Avg. No. Colonies 2 Plates		Viability Ratio		
				100 μg		500 μg										
Fraction	Pos. Control, Anthramine 100 μg	Sample 100 μg	Sample 500 μg	+S-9	-S-9	+S-9	-S-9	+S-9	-S-9	+S-9	-S-9	+S-9	-S-9	+S-9	-S-9	
<1.7 μm																
Base	30	1	8	-	-	-	-	-	-	-	-	-	-	-	-	
Acid	TNTC	53^b	111^b	-	-	-	-	-	-	-	-	-	-	-	-	
PNA	-	Not tested		-	-	-	-	-	-	-	-	-	-	-	-	
Paraffins	TNTC	0	0	-	-	-	-	-	-	-	-	312	364	1.1	1.0	
Polar Neutrals	TNTC	0	0	27	33	24	31	95	34	1.3	0.5	319	256	1.1	0.7	
>1.7 μm																
Base	TNTC	0	0	-	-	-	-	-	-	-	-	-	-	-	-	
Acid	101	0	0	-	-	-	-	-	-	-	-	-	-	-	-	
PNA	-	Not tested		-	-	92^b	83^b	460^b	267^b	6.1^b	4.0^b	400	15	1.4	0.04	
Parraffins	108	0	0	28	26	36	32	137	71	1.8	1.1	345	299	1.2	0.9	
Polar Neutrals	30	3	3	40	23	64^b	45^b	572^b	168^b	6.3^b	2.5^b	315	215	1.1	0.6	
Control	0	0	0	28	26	32	27	75	66	1.0	1.0	278	351	1.0	1.0	

[a] Well, spit and pour plate tests were performed as described in Materials and Methods. A positive mutagenic response is indicated by b. No sample available for testing is indicated by a dash (-); addition of S-9 microsomal preparation is indicated by "+S-9"; "-S-9" indicates no S-9 addition.

TABLE 22. CHEMICAL EXTRACTION AND FRACTIONATION RESULTS FROM LAKE CHARLES, LOUISIANA

Particulate sample	Particles collected (mg)	Total organics extracted (mg)	Percent organics	Amount of organics in each fraction: Acids mg	Acids %	Base mg	Base %	Nonpolar neutrals mg	Nonpolar neutrals %	PNAs mg	PNAs %	Polar neutrals mg	Polar neutrals %	Percent recovery
October 1978 (#1)														
Lake Charles, LA #1 (<1.7 μm)	747	24.6	3.3	7.2	(29.3)	0.1	(0.4)	6.1	(24.8)	2.1	(8.5)	3.7	(15.0)	78.0
Lake Charles, LA #1 (>1.7 μm)[a]	113.6	11.9	10.5	1.7	(14.3)	0.1	(0.5)	3.9	(32.8)	2.3	(19.3)	2.4	(20.2)	87.4
February 1979 (#2)														
Lake Charles, LA #2 (<1.7 μm)	2,618	401.2	15.3	170.2	(42.4)	16.3	(4.1)	49.2	(12.3)	7.1	(1.8)	157.6	(33.0)	98.6
Lake Charles, LA #2 (1.7-3.5 μm)	488	15.2	3.1	3.9	(25.7)	1.9	(12.5)	4.9	(32.2)	1.5	(9.9)	1.3	(8.6)	88.8
Lake Charles, LA #2 (>3.5 μm)	548	26.8	4.9	16.2	(60.4)	3.1	(11.6)	5.3	(14.7)	1.0	(3.7)	0.8	(3.0)	98.5

[a] The 1.7-2.25 and 3.5-20 μm particles from this sample were combined.

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and Methods (Section 4) and the crude extract subjected to fractionation with the six chemical-class scheme (Figure 2). After evaporation of the solvents with nitrogen, each chemical-class fraction was weighed, diluted with DMSO and both the chemical-class fractions and the crude extract were subjected to mutagenicity testing in the Ames/ Salmonella test. The organic content of these particulate samples ranged from 3-15%, with the average range of extractable organic compounds averaging 7% (Table 22). The greater percentage of organic compounds were fractionated into the acid, polar, and nonpolar neutral classes for the October 1978 trip and into the acid fraction for the February 1979 trip. The content for the <1.7 μm particles collected in February of 1979 was unique, in that the acid content was very high (42.4%) and the PNA content was very low (1.8%) (Table 22). This was also true for the other particle sizes for this date; the majority of organic extractables in the >3.5 μm sized particles resided in the acid fraction (60.4%). The PNA fraction was also unusually low (3.7%) in the >3.5 μm sample of February 1979. Extraction efficiency for both sites was high (78% for the lowest percent and 98.6% for the highest percentage). Higher percent efficiency of chemical extraction was achieved in the February 1979 sampling trip. This general increase in chemical extraction efficiency was probably due in part to the greater amount of particulate collected in February.

Sample size limitations for the first Lake Charles sample (Table 23) precluded dose response plate incorporation mutagenicity testing. Only the PNA fraction was tested with the plate incorporation assay and only with S9, as these conditions are required for detection of PNAs (McCann et al., 1975). Testing of the PNA fraction without S9 was not performed due to limited sample size. Activity of the PNA fraction was detected with TA98 and TA1535 in the <1.7 μm sample, but sample size limitations precluded any quantitative dose response testing. The remaining fractions were tested with the agar well diffusion test (see Appendix C). Although the agar well diffusion test was a qualitative test, the following mutagenic activity was demonstrated in this test: the acids and polar neutral fractions extracted from <1.7 μm particulate exhibited mutagenicity for Salmonella strains TA98 and TA1537; the acids were mutagenic for TA1535; and the NPN were mutagenic for TA1537 and TA1538. Tetrabromo- bis-phenol, a compound identified by GC/MS/Computer analysis in large amount in the acid fractions from this site, was also tested in the agar well test to determine if this compound was responsible for the mutagenic activity. Negative results were obtained with all strains however with this compound.

The overall results from this initial testing of the Lake Charles site indicated that the <1.7 μm particulate sample was qualitatively more mutagenic, in more chemical-classes and with more Salmonella tester strains than was the >1.7 μm particulate sample. The acids and polar neutrals were active in the <1.7 μm but not in the >1.7 μm particle sample. Both direct-acting mutagens and promutagens were also identified on the <1.7 μm sample. Quantitative pour plate tests also suggested greater mutagenic activity in the <1.7 μm PNA fraction over the >1.7 μm PNA fraction (mutagenic activity was detected in the PNA fractions but only with TA98 and S9 addition). This fact strengthens the theory of

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TABLE 23. MUTAGENESIS DATA OF PARTICULATE FRACTIONS FROM LAKE CHARLES, LOUISIANA, SAMPLE NUMBER 2566-43,44 (<1.7 µm) PARTICLES

Fraction	Total amount sample (mg)	Activity	Test	RESULTS Average number of colonies, average of two plates Ta98 500 µg	 Ta98 100 µg	 TA1535 500 µg	 TA1535 100 µg	 TA1537 500 µg	 TA1537 100 µg	 TA1538 500 µg	 TA1538 100 µg	 TA100 500 µg	 TA100 100 µg
Base <1.7 µm				No sample									
Acid[b] <1.7 µm	7.2	c,g	Well	43	1	17[b]	6	10[b]	4	5	1	4	0
NPN[b] <1.7 µm	6.1	g	Well	9	1	8	5	45[b]	0	34[b]	1	No sample	
PNA[b] <1.7 µm	2.1	Yes[e]	Pour	172[b]	Not tested[a]	168[b]	Not tested[a]	No sample		No sample		No sample	
PN[b] <1.7 µm	3.7	b,h	Well	19[b,g]	7	8	11	17[b,h]	4	No sample		No sample	
Base >1.7 µm	0	a		No sample									
Acid >1.7 µm	1.7	d	Well	17	1	10	7	4	0	No sample		No sample	
NPN >1.7 µm	3.9	d	Well	11	2	10	4	2	0	No sample		No sample	
PNA[b] >1.7 µm	2.3	b,e	Pour	97[b]	Not tested[a]	25	Not tested[a]	32	Not tested[a]	No sample		No sample	
PN >1.7 µm	2.4	d	Well	6	2	8	5	No sample		No sample		No sample	
Controls DMSO + S9	0 0		Well Pour	3 40		5 35		3 20		4 18		25 199	
Tetrabromo-bisphenol (acid)[f]	- -	d d	Well Pour	0 42	2 Not tested	10 17	2 Not tested	4 22	1 Not tested	12 18	0 Not tested	5 No sample	3 Not tested

[a] Not tested; no sample available.

[b] Positive mutagenic response.

[c] Activity did not require S9 addition.

[d] Negative respone with or without S9.

[e] Not tested in absence of S9.

[f] This compound was tested because it was identified in large amounts in the acid fractions.

[g] Direct-acting mutagenic response.

[h] Required S9 for positive mutagenic response.

more surface area per unit weight for attachment of organic compounds on smaller size particles (Chrisp et al., 1978; Commoner et al., 1978; Fisher et al., 1979b). These results indicated the importance and necessity of size-fractionation of ambient air particles before biotesting is conducted.

Mutagenicity testing for the second Lake Charles sample was more quantitative, although no toxicity determinations were performed. Only two doses (500 and 100 μg/plate) and the <1.7 μm sample for each fraction were tested. The results for this quantitative pour plate testing are given in Table 24. The total organic mixture exhibited mutagenicity for strains TA100, TA98, and TA1538. For strain TA100, the crude extract demonstrated a mutagenic response, but the response was weak (2-fold background) and was only detected at the highest dose (1000 μg/plate). The other fractions tested (acids, bases, PN, and NPN) did not exhibit mutagenic activity for strain TA100. Mutagenic activity on TA100 suggested that direct-acting base substitution mutagens may be present. The PNA fraction was positive for strain TA98 (20-times background) in the presence of S9 metabolic activation, but was not tested without S9 or with any other _Salmonella_ strains due to sample size limitations. Increasing the amount of S9 protein with the PNA fraction on TA98 caused the mutagenic activity to decrease. This result supported the findings with BAP and the PN fraction from Baton Rouge (Figures 18 and 19). For strain TA98, the crude organic extract exhibited greater mutagenic activity in the absence of metabolic activation. The basic and NPN fractions were mutagenic for TA98, but only at the highest concentration tested (500 μg/plate). The basic fraction required metabolic activation for expression of mutagenic activity with TA98, which indicated the presence of promutagens (indirect-acting mutagens). The total number of colonies increased by 2-fold (from 51-105 colonies/plate) with S9 addition for the basic fraction with TA98. The NPN activity with TA98 was not increased by S9, which also indicated the presence of direct-acting mutagens. The testing of this Lake Charles, Louisiana sample therefore demonstrated the presence of both direct- and indirect-acting frameshift mutagens, which were detected by TA98. The crude extract fraction and PN fraction were mutagenic for TA1538 without S9 addition; TA1537 did not detect any mutagens in the PN or acid fractions. A high spontaneous reversion rate for strain TA1535 rendered the assay results for this strain uninterpretable.

In summary, the qualitative results with the agar well test from the first Lake Charles sampling trip identified the major portion of mutagenic activity on the <1.7 μm particles. The second sampling trip to Lake Charles allowed more quantitative pour plate testing. Results suggested that the PNA fraction, which was responsible for only 1.8% of the total extractable organic compounds from the <1.7 μm particles, was responsible for the major portion of mutagenic activity. The chemical fractionation scheme therefore allowed identification of a highly mutagenic but minor component that was not identified when the total organic extract was tested. This concentration ability of the fractionation scheme is especially advantageous when testing complex environmental mixtures, which may contain hundreds of compounds only a few of which

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TABLE 24. MUTAGENESIS DATA OF PARTICULATE FRACTIONS FROM LAKE CHARLES, LOUISIANA 2566-91,92 (<1.7 μm). RESULTS REPORTED AS MEAN ± STANDARD DEVIATION[a] - PLATE INCORPORATION TEST

Sample fraction	Doses (per plate)[b]	MA concentration (mg protein)	TA100 +MA	TA100 -MA	TA98 +MA	TA98 -MA	TA1538 +MA	TA1538 -MA	TA1537 +MA	TA1537 -MA
Total organics[c]	1000 μg	3.0	507±17[c]	450±21[c]	127±28[c]	210±70[c]	152±28[c]	125±12[c]		
	500 μg	3.0	327±15	341±30	74±24	169±12	60±5	80±0		
Polar neutrals[c]	500 μg	3.0	401±18	387±2	129±51[c]	241±6[c]	117±6	191±12[c]	67±2	21±11
	100 μg	3.0	186±63	256±73	94±23	155±9[c]	56±8	74±12	24±10	23±9
Acids	500 μg	3.0	326±9	221±91	82±15	77±17	59±21	67±11	62±17	19±10
	100 μg	3.0	150±95	168±57	63±20	89±22	61±5	52±12	47±21	39±4
Bases[c]	500 μg	1.5	163±106	94±63	105±17[c]	51±16	69±.7	51±8		
	100 μg	1.5	197±71	276±25	77±21	66±19	56±3	46±0		
	500 μg	3.0			103±40[c]					
	100 μg	3.0			53±24					
	500 μg	6.0			58±19					
	100 μg	6.0			62±17					
Nonpolar neutrals[c]	500 μg	3.0	398±13	224±21	122±7[c]	107±45[c]	69±5	53±3		
	100 μg	3.0	365±7	279±25	93±9	88±10	60±7	37±0		
Polynuclear aromatics[c]	500 μg	1.5			1007±159[c]					
	100 μg	1.5			394±53[c]					
	500 μg	3.0			755±144[c]					
	100 μg	3.0			283±58[c]					
	500 μg	6.0			257±198[c]					
Positive control[e]	100 μg		2404±218	280±13	2309±121	1848±41	2086±5	1260±55	39±20	174±38
	10 μg		2197±682	1241±215	2074±31	1117±47	1529±136	623±27	69±6	20±6
	1 μg		1061±14	627±177	635±38	109±17	683±30	339±78	20±2	20±2
Solvent control[f]	100 μl		218±16	205±9	51±3	53±5	64±5	44±3	38±6	24±6

[a] Data are given in revertant/plate ± standard deviation.

[b] Dose refers to μg organic extract of chemical class fraction or μg crude organic extract.

[c] Mutagenic response.

[d] PNAs were not tested without S-9 due to sample size limitation.

[e] The positive controls for each strain are listed in Appendix B.

[f] The solvent utilized was 100 μl of SPECTRAL-GRADE dimethylsulfoxide (DMSO)/plate.

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are mutagens (Pellizzari, 1979). The basic and PN fractions were also mutagenic with strain TA98 but these two fractions demonstrated only 10% of the activity of the PNA fraction. The finding that the chemical fractionation scheme could identify the most mutagenic chemical-class from a complex mixture, and could also unmask mutagenic activity that would not have been detected had only the crude extract been tested, justified the fractionation of the crude organic extracts prior to biotesting. Fractionation also permitted further evaluation of activity by quantifying the total amount of a particular chemical-class within the complex crude organic extract, and allowed meaningful chemical characterization to be performed. The mutagenic activity of the Lake Charles particulate sample correlated with the signature mutagenic/carcinogenic compounds identified in the fractions (Table 45); the highly mutagenic PNA fraction contained the largest number of mutagenic compounds.

Beaumont, Texas Ambient Air Particulate Sample

The particulate sample from Beaumont, Texas was collected in March 1979. Fractionation data is contained in Table 25. It should be noted that the presence of traces of residual solvent may be responsible for the abnormally high recovery of organics from the <1.7 μm particulate sample (58%). Three size fractions were collected but only the <1.7 μm size particulate was bioassayed, due to limited sample size. The lower sample sizes obtained from this site were due to problems encountered with the MAVS during rainy periods (*e.g.*, arcing and low plate potential). This severe sample size problem also prevented chemical analysis of the particulate sample, but chemical analysis was conducted for the vapor-phase (Table 45) and known mutagenic and carcinogenic components were identified. The exact dates for this collection were March 27 to April 4, 1979 at 600 Pine Street, the location of the Texas Air Pollution Control Board. Total sampling time was 86 hr; 89,175 m^3 of air was collected and the ambient air temperature was 70°F. The primary industrial emissions in Beaumont were from refinery, petrochemical, paint and steel and metal manufacturing plants.

The small size of the Beaumont sample limited mutagenicity testing in the Ames assay to strains TA98 and TA1535 and the <1.7 μm fraction. Results are illustrated in Table 26. The PNA fraction was mutagenically active with strain TA98, both with and without S9 metabolic activation. No mutagenic activity was demonstrated with any other fraction with this strain. The acid fraction was active with S9 addition with TA1535. The cyclohexane insoluble fractions from this site was not tested due to microbial and fungal contamination.

In summary, severe size limitations precluded adequate mutagenicity testing of the Beaumont site. Strain TA98 was chosen as the priority bacterial tester strain, as this strain was the most sensitive to the mutagenic components previously detected in extractable organic material from ambient air particles. The acid fraction was also mutagenic with TA1535, which suggested the presence of base substitution mutagens. Activity with TA98 was direct-acting, which indicated the presence of frameshift mutagens. The NPN fraction was negative with TA98, TA100,

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TABLE 25. CHEMICAL EXTRACTION AND FRACTIONATION RESULTS FROM BEAUTMONT, TEXAS

				Amount of organics in each fraction										
				Acids		Base		Nonpolar neutrals		PNAs		Polar neutrals		
Particulate sample	Particles collected (mg)	Total organics extracted (mg)	Percent organics	mg	%	mg	%	mg	%	mg	%	mg	%	Percent recovery
March 1979														
Beaumont, TX (<1.7 μm)	285	150.6	52.8	15.5	(10.3)	6.3	(4.2)	50.3	(38.4)	8.5	(5.6)	6.7	(4.4)	58.0
Beaumont, TX (1.7-3.5 μm)	882	30.7	3.4	6.8	(25.4)	0.6	(2.0)	12.2	(39.7)	2.2	(7.2)	8.9	(29.0)	100.0
Beaumont, TX (>3.5 μm)	1,701	42.1	2.5	10.4	(24.7)	0.4	(0.9)	21.3	(49.9)	4.4	(11.4)	3.8	(9.0)	96.7

TABLE 26. MUTAGENESIS DATA FOR PARTICULATE ORGANIC FRACTIONS FROM BEAUMONT, TEXAS (<1.7 μm) PARTICLES - PLATE INCORPORATION TEST

Sample fraction	Dose (per plate)	MA concentration (mg protein)	TA100		TA98		TA1537		TA1535
			+MA	-MA	+MA	-MA	+MA	-MA	+MA
Total organics	NOT TESTED - ALL EXTRACTABLE ORGANICS WERE FRACTIONATED								
Polar neutrals	500 μg	3.0			58±11	54±28			75±10
	100 μg	3.0			39±8	55±7			157±10
Acids[a]	500 μg	3.0			81±10	44±10			143±52
	100 μg	3.0			55±5	48±5			272±28[a]
Bases	500 μg	0.8			-				
	100 μg	0.8			-				
	500 μg	1.5			72±32				
	100 μg	1.5			51±21				
	500 μg	3.0			-				
	100 μg	3.0			66±9				
	500 μg	6.0			68±11				
	100 μg	6.0			74±37				
Polynuclear aromatics[a,b]	500 μg	0.8			-				
	100 μg	0.8			120±14[a]				
	500 μg	1.5			100±21[a]				
	100 μg	1.5			133±7[a]				
	500 μg	3.0			-	78±0			
	100 μg	3.0			82±1	97±0[a]			
	500 μg	6.0			96±5[a]				
	100 μg	6.0			56±3				
Nonpolar neutrals	500 μg	3.0	178±13	156±16	46±7	61±15	91±40	23±2	73±5
	100 μg	3.0	180±7	153±25	54±13	46±12	51±7	41±.7	120±8
Positive controls[c]	100 μg	3.0	2278±99	2149±215	828±88	564±24	379±58	313±85	258±8
	10 μg	3.0	1825±39	1912±90	594±32	595±77	144±16	28±2	326±2
	1 μg	3.0	355±23	773±47	293±47	193±10	61±7	33±13	252±17
Solvent control[d]	100 μl	3.0	269±16	228±38	42±14	48±13	50±3	20±5	88±8

[a] Mutagenic response.

[b] PNA's were not tested without S-9 metabolic activation due to sample size limitations.

[c] Positive controls are listed in Appendix B.

[d] Negative (solvent) controls was SPECTRAL-GRADE dimethylsulfoxide (DMSO).

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TA1537, and TA1535, which correlated with the negative activity demonstrated by this fraction with other samples, and also correlated with the absence of any signature mutagenic or carcinogenic compounds in the NPN fractions (Table 45).

Houston, Texas Ambient Air Particulate Sample

Two separate sampling visits were made to Houston, Texas: the first trip on June 5, 1979 (2566-1,2) collected two particle sizes (<1.7 μm and >1.7 μm), and the second trip on June 11, 1979 collected three particle sizes (<1.7 μm, 1.7-3.5 μm and >3.5 μm). This sampling was performed on the above dates at the Jacinto Port Fuller Company. Sampling time on both dates was 94 hr; 98,000 m^3 of air were sampled. Major industries in the Houston area include: refinery and petrochemical complexes, paint, chemical and steel manufacturing operations, and water treatment corporations.

Extraction and fractionation results for these two sampling trips are contained in Tables 27 and 28. Total amount of particulate sample was significantly lower than usual (0.3 gm for each particle size) on the June 5, 1979 trip; the second trip on June 11, 1979 collected 3.0 gm. Consequently, the testing of the first sample was conducted with the qualitative agar well test due to limited sample size; the larger amount of particulate sample from the second trip allowed quantitative plate incorporation testing in the Ames/*Salmonella* mutagenicity assay.

The percentage of total organic extract obtained from the Houston, Texas particulate sample ranged from 3.6-29.8%. It should be realized that the determination of the amount of organics extracted from particulate samples was an approximation. The presence of trace amounts of solvents which were not removed by nitrogen could create a small weighing error. However, the use of more stringent conditions (*e.g.*, vacuum treatment) to remove all of the solvent could create the undesirable loss of semi-volatile materials (*e.g.*, dodecane, naphthalene, etc). Thus, the higher apparent organic content (29.8%) of the >1.7 μm size class (Table 27) may be an artifact generated by the necessary use of wet rather than dry weight. This artifact may also account for the lower percent recovery (47.2% by weight) of the various chemical fractions of the >1.7 μm particle class from this first Houston sampling trip. The mass obtained from the the total mass of the individual chemical-classes may be a more accurate expression of the percentage of total extractable organic compounds.

The organic fractions from the <1.7 μm and the >1.7 μm fractions from the first sampling trip exhibited qualitative differences (Table 27). Higher concentrations of nonpolar neutrals (NPN) and polynuclear aromatic hydrocarbons (PNA) were found in the <1.7 μm fraction. When results from the <1.7 μm fraction from sampling visit #1 (Table 27) was compared with <1.7 μm fraction from the second sampling visit (Table 28), the second visit exhibited a higher percent of acids and a slightly lower percent of PNA content. Scanning electron micrographs and energy dispersive X-ray spectra of the particulates from both sampling visits

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TABLE 27. EXTRACTION/FRACTIONATION RESULTS FROM HOUSTON, TEXAS (2566-1,2) (<1.7 µm) SAMPLE 1

Particulate Collected (g)	Extracted Organics (mg)	Percent Extractable Organics
0.3374	19.3	5.7%
Fraction	**Wgt. (mg)**	**% of Total Extract**
Acids	1.0	5.18
Bases	0.6	3.10
Polynuclear Aromatics	3.2	16.58
Polar Neutrals	9.9	51.29
Non-Polar Neutrals	4.0	20.73
	18.7 Total Recovery	96.88% Total Recovery

EXTRACTION/FRACTIONATION RESULTS FROM HOUSTON, TEXAS (2566-1,2) (>1.7 µm)

Particulate Collected (g)	Extracted Organics (mg)	Percent Extractable Organics
0.2901	86.4	29.8%
Fraction	**Wgt. (mg)**	**% of Total Extract**
Acids	0.8	0.92
Bases	1.2	1.38
Polynuclear Aromatics	2.4	2.77
Polar Neutrals	33.2	38.43
Non-Polar Neutrals	3.2	3.7
	40.8 Total Recovery	47.2% Total Recovery

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TABLE 28. EXTRACTION/FRACTIONATION RESULTS FROM HOUSTON, TEXAS (2566-105) (<1.7 μm), SAMPLE II

Particulate Collected (g)	Extracted Organics (mg)	Percent Extractable Organics
3.043	110.2	3.6%

Fraction	Wgt. (mg)	% of Total Extract
Acids	20.4	18.51
Bases	2.8	2.54
Polynuclear Aromatics	12.2	11.07
Polar Neutrals	38.0	34.48
Non-Polar Neutrals	20.9	18.96
	94.3 Total Recovery	85.56% Total Recovery

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demonstrated that the particles were generally homogenous within a given size class, but mixing did occur between the different particles sizes. The results suggested that the size classification of the MAVS was not absolute.

The majority of the particulate collected from the second trip was in the <1.7 μm size class (3,043 mg) with an organic content of 3.6% by weight (Table 28). The second sampling visit also included collection of volatile organics (Table 45). Sampling and identification of volatile organics was achieved with two separate Tenax® GC polymeric sorbent cartridges and subsequent thermal desorption and GC/MS/Computer analysis. Table 45 illustrates the signature mutagenic/carcinogenic organic compounds identified in the chemical-classes from each size classified particulate sample.

Initial mutagenicity testing results of the first particulate sample from Houston, Texas was conducted on June 5, 1979 are contained in Table 29. Due to limited sample size, the agar well assay was utilized with the developed bioassay priority scheme (Table 4). The nonextracted particulate and cyclohexane insoluble fraction (CHI) for the <1.7 μm and >1.7 μm sample were tested but were heavily contaminated with mold and bacteria and were therefore incompatible with bioassay procedures. The <1.7 μm sample exhibited significant mutagenic activity with TA98 in the organic acids and PNA fractions (Table 29). The PN class of the <1.7 μm particles was also mutagenic with strain TA98 and with strain TA1538. The activity in the PN and acid fractions did not require S9 metabolic activation. The PN fraction in the <1.7 μm particulate sample increased from 45-101 revertants when sample size was increased from 100 to 500 μg/plate with TA1538. Consequently, both direct- and indirect-acting mutagens were detected in the acid and PN fractions of the <1.7 μm particles from Houston, Texas.

Testing of the >1.7 μm particles from the first sampling trip to Houston (Table 29) suggested that the total extractable organics from the >1.7 μm particulate sample were not mutagenically active with any of the five tester strains in the agar well test. However, the PNA fraction was active with TA1535 and S9 addition. Further testing of this >1.7 μm sample (Table 30) with the agar well test and the pour plate assay and Salmonella strain TA98 suggested that the acid and PNA fractions were active. The total extractable organics from the >1.7 μm particulate sample did not demonstrate mutagenic activity with either the pour plate or the agar well assay. This result demonstrates the utility of the chemical fractionation scheme; mutagenic activity would have not been detected if only the total extractable organic fraction had been tested. The negative mutagenic potential of the crude organic fraction was detected with both assay techniques and was repeated on different assay days (see Tables 29 and 30). In addition, the inability of water insoluble PNA compounds to be detected in the agar well assay was also demonstrated, as the PNA fraction was only active in the pour plate assay. This diffusivity limitation was the reason the agar well assay was not utilized with PNA fractions (see Bioassay Priority Scheme, Table 2).

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TABLE 29. INITIAL MUTAGENESIS DATA OF PARTICULATE FRACTIONS FROM HOUSTON, TEXAS (2566-1,2) (<1.7 μm) SAMPLE I (JUNE 5, 1979)[a]

		Average of two plates - background not subtracted									
		TA98		TA1537		TA1538		TA100			Did activity require
Particulate	Type Plate	500 μg	100 μg	500 μg	100 μg	500 μg	100 μg	500 μg	100 μg	Total Amt. sample (mg)	S9
Base (<1.7 μm)	Well	29	2	No sample		No sample		No sample		0.6	-
Acid (<1.7 μm)	Well	161[b]	93[b]	No sample		No sample		No sample		1.0	No
PNA (<1.7 μm)	Pour	141[b]	NT[c]	28	NT[C]	No sample		No sample		3.2	Yes
PN (<1.7 μm)	Well	63[b]	37[b]	11	4	101[b]	45[b]	14	9	9.9	No
NPN (<1.7 μm)	Well	17	5	3	1	No sample		No sample		4.0	
Total Extractable organics (>1.7 μm)	Well	8	13	9	5	6	0	12	5	40.7	
PNA >1.7	Pour										
Controls DMSO	Well	9	5	3	4	4	3	9	7	0	
+ S9	Pour	45		15		14		75			

[a] The nonextracted particulate and the cyclohexane insoluble (INT) fractions for both particle size (<1.7 and >1.7 μm) could not be tested due to bacterial and funal contamination. Insufficient sample was available for testing with TA1535.

[b] Mutagenic.

[c] C = NT = Not tested.

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TABLE 30. MUTAGENESIS DATA OF PARTICULATE FRACTIONS FROM HOUSTON, TEXAS (2566-1,2) (>1.7 μm) SAMPLE I (JUNE 5, 1979) WITH SALMONELLA STRAIN TA98

	Average of Two Plates - Strain TA98						
Particulates	Type Plate	500 μg/plate	100 μg/plate	Type Plate	500 μg + S-9	Total Amt. Samples (mg)	Did Activity Require S-9
>1.7 μm Total Extractable Organics	Well	8	7	Pour	39	61.7	
Base	Well	7	5	Pour	No Sample	1.2	
Acid	Well	63[a]	50[a]	Pour	No Sample	0.7	No S-9 Required
PNA	Well	16	9	Pour	130[a]	2.4	Not Tested Without S-9
NPN	Well	4	6	Pour	40	3.2	
PN	Well	5	5	Pour	40	33.2	
Solvent Control[b]	Well Pour	5	5		50	-	

[a] Mutagenic response.

[b] 100 μl DMSO.

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The second sampling trip to Houston, Texas was performed on June 11, 1979 (Table 31). The <1.7 µm particulate sample was fractionated and tested with multiple S9 concentrations (0.8, 1.5, 3.0 and 6.0 mg of S9 protein/plate). Salmonella strain TA98 was the first strain utilized in this plate incorporation testing. The PNA fraction was active both with and without S9 addition; revertants colonies increased from 125-408 with the addition of 3.0 mg of S9 protein. It should also be noted that activity in the PNA fractions was only detected at 500 µg/plate and not at 100 µg/plate. The results again demonstrated the necessity for large sample amounts for dose responsive bioassays of ambient air particle. The PNA fraction was also active with TA1535. The PN fraction was active with TA98; activity was similar to the PNA fraction without S9 addition, but activity for the PN fraction did not increase significantly with S9 addition. The NPN fraction was marginally active (mutagenic ratio of slightly greater than 2.0) with TA98; variation of S9 protein content did not effect the mutagenic response. The acids and basic fractions with TA98 were also marginally mutagenic. The total extractable organic fraction was not active with TA98, again suggesting the importance of chemical fractionation when testing complex environmental mixtures.

The acid fraction with TA1535 (Table 31) demonstrated mutagenic activity that required S9 addition and activity was detected at 500 and 100 µg/plate. Activity of the PN fraction with TA1535 increased 3-fold when the S9 concentration was increased from 3.0-6.0 mg of S9 protein plate. This result suggested the importance of testing complex environmental mixtures with multiple S9 concentrations. Direct-acting mutagens in the PN fraction were also detected with TA1535; 1.5 mg of S9 protein inhibited the positive response demonstrated in the absence of S9 (174 revertant colonies without 1.5 mg of S9 versus 64 revertants with S9 addition). These results suggest the presence of both direct- acting mutagens and promutagens in this Houston, Texas sample.

In summary, for both sampling visits to Houston, Texas, mutagenic potential was demonstrated for the polynuclear aromatic hydrocarbons (PNA) and the polar neutral (PN) fractions. In both sampling visits, the organic acid fraction also exhibited mutagenic potential. Unfortunately, insufficient sample collected during the first sampling visit limited bioassay determinations and precluded correlations between the two samples. These results demonstrated the unmasking of mutagenic activity in the crude organic extract with chemical fractionation. The effect of multiple S9 concentrations on the number of revertant colonies detected was also demonstrated.

Upland, California Ambient Air Particulate Sample

The Upland, California ambient air particulate sample was collected on November 11, 1977 and size fractionated into three classes: <1.7 µm, 1.7-3.5 µm, and >3.5 µm. Major industries in the Upland, California area include petrochemical and refinery operations. Extraction and fractionation information for the Upland site is contained in Tables 32 and 33. Sample amounts were 650 mg for the <1.7 µm and >3.5 µm particles;

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TABLE 31. MUTAGENESIS DATA OF PARTICULATE FRACTIONS FROM HOUSTON, TEXAS (2566-105) SAMPLE II (JUNE 11, 1979) (<1.7 µm) WITH PLATE INCORPORATION ASSAY, (Results reported in mean value of triplicate plates ± SD)

Sample Fraction	Dose µg/plate	MA Concentration mg protein	TA100		TA98		TA1537		TA1535	
			+MA	-MA	+MA	-MA	+MA	-MA	+MA	-MA
Total Extractable Organics	1000 µg	3.0			52±10	51±2				
	100 µg	3.0			35±.7	–				
Polar Neutrals	500 µg	1.5					72±8	51±6[b]	64±18	174±26[b]
	100 µg	1.5					27±21	62±18[b]	71±17	72±35
	500 µg	3.0	298±51	202±4			70±16		80±10	
	100 µg	3.0	251±11	203±27	174±13[b]	104±3[b]	54±25		98±50[b]	
	500 µg	6.0			54±13	95±10	31±6		317±112[b]	
	100 µg	6.0					18±2		203±21[b]	
Acids	500 µg	3.0	196±4	190±12	88±7[b]	64±17	48±11	33±9	150±31[b]	68±15
	100 µg	3.0	228±24	182±16	50±9	48±10	36±18	32±9	200±38[b]	69±16
Bases	500 µg	3.0			88±18[b]	58±17				
	100 µg	3.0			42±15	54±8				
Polynuclear Aromatics	500 µg	3.0			408±54[b]	125±13[b]			196±46[a,b]	90±8[a,b]
	100 µg	3.0			81±7	94±17			111±28[a]	80±21[a]
Non-Polar Neutrals	500 µg	0.8			96±11[b]	67±4				
	100 µg	0.8			90±6[b]	70±11				
	500 µg	1.5			94±5[b]					
	100 µg	1.5			65±4					
	500 µg	3.0			82±19				65±7[a]	53±6[a]
	100 µg	3.0			77±13				69±7[a]	71±17[a]
	500 µg	6.0			91±15[b]					
	100 µg	6.0			74±21					
Positive Controls[c]	100 µg	3.0	2278±99	2149±215	828±88	564±24	379±58	313±85	350±8	1787±19[a]
	10 µg	3.0	1825±39	1912±90	594±32	595±77	114±16	28±2	182±2	1687±77[a]
	1 µg	3.0	355±23	773±47	293±47	193±10	61±7	33±14	197±17	793±47[a]
Solvent Control	0.1 ml DMSO	3.0	269±16	228±38	42±14	48±13	50±3	20±5	46±9	42±3

[a] Solvent control +MA 88±8
-MA 115±73.

[b] Mutagenic response.

[c] Positive controls are listed in Appendix B.

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TABLE 32. EXTRACTION/FRACTIONATION RESULTS FROM UPLAND, CALIFORNIA (<1.7 µm) PARTICLES

Particulate Collected (g)	Extracted Organics (mg)	Percent Extractable Organics
0.5661	32.7	4.9%
Fraction	**Wgt. (mg)**	**% of Total Extract**
Acids	2.9	8.86
Bases	1.0	3.05
Polynuclear Aromatics	1.6	4.89
Polar Neutrals	15.8	48.32
Non-Polar Neutrals	6.5	19.88
	27.8 Total Recovery	85% Total Recovery

EXTRACTION/FRACTIONATION RESULTS OF UPLAND, CALIFORNIA (>3.5 µm) PARTICLES

Particulate Collected (g)	Extracted Organics (mg)	Percent Extractable Organics
0.6412	35.9	5.6%
Fraction	**Wgt. (mg)**	**% of Total Extract**
Acids	1.1	3.06
Bases	0.7	1.95
Polynuclear Aromatics	2.5	6.96
Polar Neutrals	16.0	44.57
Non-Polar Neutrals	9.4	26.18
	29.7 Total Recovery	82.72% Total Recovery

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TABLE 33. EXTRACTION/FRACTIONATION RESULTS FROM UPLAND, CALIFORNIA (1.7-3.5 μm) PARTICLES

Particulate Collected (g) 0.2857	Extracted Organics (mg) 17.5	Percent Extractable Organics 6.1%
Fraction	**Wgt. (mg)**	**% of Total Extract**
Acids	1.8	10.28
Bases	0.6	5.84
Polynuclear Aromatics	2.0	11.42
Polar Neutrals	6.3	36.00
Non-Polar Neutrals	4.7	26.86
	15.4 Total Recovery	90.4% Total Recovery

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only 286 mg of particulate sample was collected for the 1.7-3.5 μm particles. Total extractable organics ranged from 4.9-6.1%; total recovery for all size classes was above 83%. The total amount of sample in each class precluded dose response plate incorporation mutagenicity testing; consequently, the agar well diffusion assay was utilized. The only chemical-class that contained more than 10 mg of sample was the PN fractions of the <1.7 and >3.5 μm sized particles.

Due to limitations imposed by sample size, all of the extracted crude organic fraction from Upland, California, was subjected to fractionation prior to bioassay. The agar diffusion well test was utilized for determinations of mutagenic activity. Table 34 contains the results of mutagenicity testing of the three size-classified particles. The <1.7 μm particulate sample exhibited a significant mutagenic response in the organic acid, polynuclear aromatic hydrocarbon, and polar neutral fractions. The organic acid fraction was mutagenic with TA98, TA1537, and TA1538 without S9 addition, which suggested the presence of direct-acting frameshift mutagens. The polar neutral fraction was mutagenic for TA98, TA1537, and TA1538, particularly at 500 μg sample/plate, which again suggested the presence of frameshift mutagens. The polynuclear aromatic fraction was only tested with Salmonella strain TA98 and S9 addition due to the limited sample size. Nonetheless, 242 revertant colonies were detected with 500 μg of sample and S9 addition in the PNA fraction of the <1.7 μm particles.

The 1.7-3.5 μm size classified particulate sample exhibited significant mutagenic potential when the acid, PNA, and polar neutral fractions were bioassayed. At 500 μg sample/plate, the organic acid fraction gave a positive mutagenic response with strain TA98 without S9, which suggested the presence of direct-acting frameshift mutagens. The PN and NPN fractions did not require S9 microsomal activation for mutagenic activity with strain TA98. Positive mutagenic responses for the PN fraction also was obtained with strain TA1537, while a negative response was observed with strain TA1535, which suggested the presence of frameshift mutagens in the polar neutral (PN) fraction. The nonpolar neutral (NPN) fraction was also mutagenic without S9 addition, but only with TA98.

The agar diffusion well test was employed with all of the chemical fractions of the >3.5 μm particles, except for the PNA fraction which was assayed by the pour plate method. The results in Table 34 indicated that only the organic acid and PN fractions exhibited mutagenic potential when the >3.5 μm particulates were bioassayed. The polar neutrals exhibited marginal mutagenic activity for strain TA1538. The base and the NPN fractions were not mutagenic.

In summary, no quantitative response mutagenicity information was generated for the total extractable organics in any of the three size classes due to sample size limitations. After chemical fractionation of the crude organic extract, the organic acid fractions were mutagenic in all three size classes. The PNA fraction was mutagenic for the three smaller size classes. The PN fraction also exhibited mutagenic potential in all three size classes. The general trend for this Upland, California

TABLE 34. MUTAGENESIS DATA OF PARTICULATE FRACTIONS FROM UPLAND, CALIFORNIA (2566-5354; 5859; 5556)

Fraction	Total Sample	Was S-9 Required for Activity	Type Plate	Average Number of Colonies: TA98 500 μg	TA98 100 μg	TA1535 500 μg	TA1535 100 μg	TA1537 500 μg	TA1537 100 μg	TA1538 500 μg	TA1538 100 μg	TA100 500 μg	TA100 100 μg
<1.7 μm													
Base	1.0 mg		WELL	6.0	9.0	No Sample →							
Acid	2.9 mg	NO	WELL	17.0[a]	18.0[a]	2.0	2.0	25.0[a]	25.0[a]	15.0[a]	No Sample →		
PNA	1.6 mg	YES	POUR	242.0[a]	Not Tested	No Sample →							
PN	15.8 mg	NO	WELL	6.0	15.0[a]	3.0	2.0	14.0[a]	8.0	25.0[a]	15.0	16.0	9.0
NPN	6.5 mg		WELL	5.0	6.0	3.0	3.0	2.0	2.0	1.0	4.0	6.0	10.0
1.7-3.5 μm													
Base	0.6 mg		WELL	28.0	No Sample →								
Acid	1.8 mg		WELL	98.5[a]	75.0[a]	14.0	6.0	7.0	2.0				
PNA	2.0	YES	POUR	32.5[a]	28.0[a]	7.5	5.0	No Sample →					
PN	6.3 mg		WELL	97.5[a]	59.0[a]	3.5	6.5	19.0[a]	14.0[a]				
NPN	4.7 mg		WELL	15.0	46.0[a]	4.0	6.5	2.0	3.0				
>3.5 μm													
Base	0.7 mg		WELL	3.0	3.0	No Sample →							
Acid	1.1 mg		WELL	77.0[a]	46.5[a]	No Sample →							
PNA	2.5 mg		POUR	-	-	82.0[a]	No Sample →						
PN	16.5 mg		WELL	5.0	9.0	3.0	4.0	6.0	5.0	8.0[a]	15.0[a]	No Sample →	
NPN	9.4 mg		WELL	3.0	4.0	5.0	3.0	4.0	3.0	1.0	1.0	9.0	5.0
Control	0		WELL	4.0	4.0	3.0	3.0	3.0	3.0	2.0	2.0	10.0	10.0
DMSO + S9[b]	0		POUR	51.0	51.0	-	-	-	-	-	-	-	-

[a] Positive mutational response.

[b] DMSO = 100 μl

sample was that the acid, PNA, and PN fractions were mutagenically active, and the qualitative mutagenic activity of the two small sized classes (<1.7 and 1.7-3.5 µm) was greater than the larger sized prticles (3.5-20.0 µm). These results support the findings of other investigators (Commoner et al., 1978; Fisher et al., 1979); that the smaller sized particles have more surface area per unit weight, which provides more surface area for attachment of organic compounds.

Elizabeth, New Jersey Ambient Air Particulate Sample

The ambient air particles from Elizabeth, New Jersey were collected from September 19, 1978 to September 22, 1978 at the Exxon Bayway-Linden Generating Station. Ambient air temperature was 78°F and 81,21/m^3 of air was sampled. Sampling time was 75.9 hr. Major pollution sources in the Elizabeth, New Jersey area include: petrochemical and refinery operations, chemical manufacturing plants, and heavy industrial and commuter motor vehicle traffic. The sampling site was adjacent to the New Jersey Turnpike, at Exit 13.

Extraction/fractionation results for the three size classified particulate samples (<1.7 µm, 1.7-3.5 µm, >3.5 µm) collected from Elizabeth, New Jersey are contained in Tables 35 and 36. Considerable mixing occurred between the different size classes, which indicated the failure of the MAVS to absolutely discriminate sizes. The organic content (percent by weight) of the particulate samples were as follows: <1.7 µm (13.4%); 1.7-3.5 µm (9.2%); and >3.5 µm (6.9%). The percent extractable organics increased with decreasing size of the particles; particulate sample for each class was approximately 1.3 gm. Since the <1.7 µm particles have a greater surface area per total weight for adsorption of organics, it should contain the largest organic content per unit particle mass. Total recovery of the organics after fractionation was the same for each size class (78%). Identification of compounds in the particulate and vapor-phase organic samples was accomplished with thermal desorption and GC/MS/Computer analyses (Table 45). A greater number of signature mutagens and carcinogens were identified with the <1.7 µm particles.

As a consequence of insufficient sample, both the agar diffusion well test and the plate incorporation test were employed to determine mutagenic potential (Table 37). The organic acid fraction from the <1.7 µm particles was mutagenic with all five of the Salmonella strains, both with and without S9 addition, which indicated the presence of both frameshift and base substitution mutagens. The polynuclear aromatic hydrocarbons (PNA) were mutagenic with S9 activation for strains TA1537, TA1538, and TA100. The PNAs were not tested without S9. The polar neutral (PN) fraction demonstrated mutagenic potential with TA98, TA1537, TA100, and TA1538 but not with strain TA1535. These results suggested the predominance of frameshift mutagens in the polar neutral and polynuclear aromatic fractions of the <1.7 µm particulate sample. The nonpolar neutrals (NPN) exhibited no mutagenic potential with any of the five bacterial tester strains. The total extractable organics in the <1.7 µm particulate sample were bioassayed for mutagenicity, but they were nonmutagenic with all five strains.

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TABLE 35. EXTRACTION/FRACTIONATION RESULTS FROM ELIZABETH, NEW JERSEY (<1.7μm)

Particulate Collected (g)	Extracted Organics (mg)	Percent Extractable Organics
1.3471	180.1	13.4%

Fraction	Wgt. (mg)	% of Total Extract
Acids	8.5	4.72
Bases	0.5	0.28
Polynuclear Aromatics	11.1	6.16
Polar Neutrals	34.6	19.2
Non-Polar Neutrals	86.2	47.86
	140.9 Total Recovery	78.22% Total Recovery

EXTRACTION/FRACTIONATON RESULTS OF ELIZABETH, NEW JERSEY (1.7-3.5μm)

Particulate Collected (g)	Extracted Organics (mg)	Percent Extractable Organics
1.3021	119.7	9.2%

Fraction	Wgt. (mg)	% of Total Extract
Acids	10.4	8.68
Bases	0.4	0.33
Polynuclear Aromatics	9.6	8.02
Polar Neutrals	26.1	21.8
Non-Polar Neutrals	47.6	39.76
	94.1 Total Recovery	78.59% Total Recovery

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TABLE 36. EXTRACTION/FRACTIONATION RESULTS FROM ELIZABETH, NEW JERSEY (>3.5 μm)

Particulate Collected (g)	Extracted Organics (mg)	Percent Extractable Organics
1.4350	99.4	6.9%
Fraction	**Wgt. (mg)**	**% of Total Extract**
Acids	5.5	5.53
Bases	0.2	0.20
Polynuclear Aromatics	6.0	6.03
Polar Neutrals	13.4	13.5
Non-Polar Neutrals	52.6	52.91
	77.7 Total Recovery	78.17% Total Recovery

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TABLE 37. MUTAGENESIS DATA OF PARTICULATE FRACTIONS FROM ELIZABETH, NEW JERSEY

Fraction	Total Sample	S9 Required	Test	TA98 500 mg	TA98 100 mg	TA1535 500 mg	TA1535 100 mg	TA1537 500 mg	TA1537 100 mg	TA1538 500 mg	TA1538 100 mg	TA100 500 mg	TA100 100 mg
<1.7 µm													
Base	0.5 mg	No	Well	44[a]	36[a]	No Sample		No Sample		No Sample		No Sample	
Acid	8.5 mg	No	Well	69[a]	69[a]	96[a]	7[a]	56[a]	31[a]	35[a]	24[a]	38[a]	34[a]
PNA	11.1 mg	Yes	Pour	NT[b]		NT[b]		262[a]	NT[b]	335[a]	NT[b]	565[a]	NT[b]
PN	34.6 mg	Yes	Pour Well	290[a]	60	7	13	28[a]	23[a]	19[a]	13[a]	382[a]	179
NPN	86.2 mg		Pour Well	79	74	6	5	-[c]	-[c]	-[c]	-[c]	213	156
1.7-3.5 µm													
Base	0.4 mg		Well	NT[b]	43[a]	No Sample		No Sample		No Sample		No Sample	
Acid	10.4 mg	No	Pour Well	331[a] 57[a]	84 41[a]	5	5	37[a]	27[a]	29[a]	22[a]	34[a]	27[a]
PNA	9.6 mg	Yes	Pour	NT[b]		NT[b]		178[a]	NT[b]	218[a]		450[a]	NT[b]
PN	26.1 mg	Yes	Pour Well	286[a]	80	4	3	22	18	21	19	497	238
NPN	47.6 mg		Pour Well	70	62	-[c]	-[c]	-[c]	-[c]	-[c]	-[c]	245 -[c]	236 -[c]
>3.5 µm													
Base	0.2 mg		Well	-[c]	31	No Sample		No Sample		No Sample		No Sample	
Acid	5.5 mg		Well	32	9	6	3	6	7	No Sample		No Sample	
PNA	6.0 mg	Yes	Pour	NT[b]		NT[b]		200[a]	NT[b]	No Sample		No Sample	
PN	13.4 mg	Yes	Pour Well	202[a]	70	4	2	12	7	16	15	289	NT[b]
NPN	52.6 mg		Pour Well	37	4	6	3	8	3	5	3	168	161
Total Organics (<1.7 µm)	18.7 mg		Pour Well	101 28	49 26	3	7	5	3	9	12	224 17	177 14
Total Organics (1.7-3.5 µg)	15.5 mg		Pour Well	Toxic		4	3	5	3	21[a]	13	265 15	182 17
Total Organics (>3.5 µm)	14.5 mg		Pour Well	Toxic	79	5	5	16	9	21[a]	8[a]	Toxic 20	16
Control Well (DMSO + AM)				20		7		8		2.5		8	
Control Pour (DMSO + AM)				63		-[b]		25		25		198	
DMSO Control				57		-[b]		19		22		155	

[a] Positive mutagenic response.

[b] Not tested.

[c] Negative mutagenic response.

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The organic acids of the 1.7-3.5 μm particles were mutagenic for TA98, TA1537, TA1538, and TA100 but were nonmutagenic with TA1535, which suggested the presence of frameshift mutagens. The polynuclear aromatic hydrocarbons (PNA) were mutagenic for strains TA100, TA1537, and TA1538. Note the decrease in the number of revertants with the 1.7-3.5 μm PNAs when compared to the <1.7 μm PNAs (Table 37). The polar neutral fraction of the 1.7-3.5 μm particulate sample was mutagenic for TA98, and marginal mutagenicity was detected with bacterial tester strain TA100, TA1537, and TA1538. The polar neutrals were nonmutagenic in the agar well assay when bacterial strain TA1535, TA1537, and TA1538 was utilized in bioassay. The <1.7 μm PN fraction was also more mutagenic than the 1,7-3.5 μm PN fraction. The nonpolar neutrals (1.7-3.5 μm) were nonmutagenic. The crude extractable organic fraction of the 1.7-3.5 μm particles was mutagenic with TA1538 and nonmutagenic with all other tester strains.

For the >3.5 μm particulate sample, a general decrease in mutagenicity was observed when results from these particles were compared to the other fractions (<1.7 and 1.7-3.5 μm). The polynuclear aromatic hydrocarbons and the polar neutrals were again mutagenic, but the total number of revertants decreased. The organic acids, bases, and nonpolar neutrals were nonmutagenic. Total extractable organics for the >3.5 μm particulate sample were mutagenic with only TA1538 and were nonmutagenic with the other four Salmonella strains utilized.

In summary, the total extractable organics were not significantly mutagenic for any of the three size-fractionated particulate samples tested in the Ames/Salmonella assay. Toxicity was also present in the crude organic fractions. Upon fractionation of the crude extract, toxicity was removed and mutagenicity was unmasked. Consequently, these results suggested both the usefulness and necessity of chemical fractionation for mutagenic analysis of complex environmental mixtures such as ambient air.

Results suggested that the PNA fraction was mutagenic for all three particle sizes; mutagenic activity decreased with increasing particle size. The PNA fraction was only tested with S9 addition; therefore no conclusions can be reached with regard to S9 requirements. However, the PN fraction required S9 addition for activity. The PN fraction also exhibited decreased mutagenic activity with increasing particle size. Mutagenic activity of these two fractions (PNA and PN) correlated with the signature mutagens/carcinogens identified in these fractions (Table 45). The NPN fractions did not demonstrate mutagenic activity with any of the five tester strains in any particle class. The acid fraction was most active in the <1.7 μm particulate sample, and the base fraction could not be evaluated properly due to sample size limitations. Overall, the <1.7 μm particulate sample was the most mutagenic of the three particulate samples tested. The Elizabeth, New Jersey sample was the most comprehensively tested under this program. Mutagenic potential and trends demonstrated with this particulate sample were also demonstrated with the other six particulate samples. These trends will be examined more fully in the Discussion (Section 6).

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MUTAGENICITY TESTING OF VAPOR-PHASE ORGANIC FIELD SAMPLES AND PURE COMPOUNDS

Mutagenicity testing of vapor-phase organic field samples was not accomplished under this program due to the lack of an appropriate testing technique for the Ames/Salmonella assay. However, one set of vapor-phase organic samples was initially tested with the hollow-fiber filter technique. The testing conditions were as follows:

Strain	TA98
S9	3 mg of protein/plate
Solvent	Ethylene glycol
Fiber type	PM-2 or PM-5
Fibers/plate	1 and 3

No positive results were obtained. It was later learned from Mr. R. Testa (Romicon Corporation, personal communication) that both DMSO and ethylene glycol solvents were incompatible with the fibers because solvents would seal the pores in the fibers. This fact explained the negative results obtained in the initial hollow-fiber filter test with known mutagens (Table 5). The DMSO or ethylene glycol caused the fibers to swell and the fiber pores to seal, which prevented diffusion of the test sample from the fiber. The fiber filter technique was therefore discarded as a result of diffusivity limitations of the test compounds from the fiber into the agar, as discussed earlier in the Development of Methods (Section 4).

After completion of experiments to evaluate vapor test procedures and obtain maximum sensitivity with the preincubation technique (Development of Methods, Section 4), further experimentation was performed to evaluate the efficiency of the preincubation technique for detection of the mutagenicity of pure vapor-phase organic compounds. This evaluation had to be performed before any actual field testing could begin. It was planned that the preincubation technique would be validated with known positive mutagenic vapor-phase compounds, and then be applied to actual vapor-phase organic field samples if reproducible mutagenic activity could be demonstrated. A literature search was therefore performed and known mutagenic vapor-phase organic compounds were identified (Table 38). Many of the vapor-phase organic compounds were identified as weakly mutagenic, but this may be due to an inappropriate method of detection rather than an actual low mutagenic potential. Based on this solubility table, the solvent-of-choice for vapor-phase organic mutagens was empirically determined to be a mixture of glycerin/water/95% ethanol in a 2:2:1 v/v ratio. One hundred microliters of this solvent mixture per plate were utilized in all experiments. Glycerin was chosen because it was compatible with and was utilized in the thermal desorption and cryogenic freezing of field samples and pure compounds (see Materials and Methods - Section 4). Water was utilized for all aqueous-soluble vapor-phase compounds. Ethanol was utilized because most of the volatile compounds chosen for this study were soluble in 95% ethanol (Table 38).

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Toxicity was not present with these three solvents at the concentrations utilized in the bioassay. Dimethylsulfoxide (DMSO) could not be utilized in this study as it was incompatible with thermal desorption/cryogenic freezing and therefore DMSO would never be employed in actual vapor-phase field sampling.

Mutagenic vapor-phase organic compounds were selected from the list of compounds in Table 38 and were tested in the Ames/Salmonella assay with three different techniques. The standard plate incorporation technique (Ames et al., 1975b) was performed as a baseline for comparison of sensitivity/specificity. This technique utilized both stationary-phase cells (standard Ames protocol) and log-phase cells. The third technique utilized in this study was the preincubation technique of Yahagi et al. (1975), as modified for this study (see Materials and Methods - Section 4). These three techniques were utilized with each volatile mutagen tested. Both toxicity assays (via clonal survival) and mutagenicity assays (via revertant colonies) were determined for each technique, at all sample and control concentrations on the same day. The experimental protocol permitted the evaluation of a sensitivity increase/decrease as a function of growth phase of cells (log versus stationary) and technique (preincubation versus pour plate).

The Salmonella strain utilized for the study of these pure vapor-phase mutagenic compounds was TA100, since this strain was indicated as the most sensitive tester strain for these vapor-phase compounds (McCann et al., 1975). Results suggested that the following vapor-phase compounds were mutagenically active with one or more of the above techniques: butylene oxide, propylene oxide, ethylene dibromide, styrene oxide, ethylene oxide, and vinylidine chloride. Results for these compounds are included in this section for each of the three techniques utilized. Other compounds that were tested with the three techniques and TA100, but which were not mutagenically active included: epichlorhydrin, methyl iodide, methylene chloride, and butadiene diepoxide. These negative results are not included in this report. Epichlorohydrin required S9 activation for detection of mutagenic activity (Bridges, 1978), and methylene chloride is an active mutagen in the gaseous state (Claxton, EPA/RTP, personal communication). This pilot study utilized only TA100, without S9 addition, in liquid culture. A future study should include all five common tester strains (TA98, TA100, TA1535, TA1537, TA1538) both with and without S9 addition, in both liquid/gaseous states.

Tables 39 through 44 contain the total revertants per plate for each of the six mutagenically active compounds with each technique, at each concentration tested. For clarity, tables and graphs follow the discussion of each compound. Included on each table is the standard deviation at each sample concentration. Spontaneous back mutation rates (SBMR) for each of the three techniques were determined and subsequently subtracted from the actual number of observed revertants per plate (Revertants - SBMR column). Revertants per survivor were then calculated by normalizing all revertants to 100% survival (revertants/survivor column). This calculation was possible since toxicity experiments were

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TABLE 38. MODEL VAPOR-PHASE COMPOUNDS FOR PREINCUBATION VALIDATION STUDY

Compound	Reference (page)[a]	MW	BP(°C)	Solubility	Mutagenicity Results[b,c]
Butadienediepoxide	Merck 418	86.09	140-142	Aqueous soluble with hydrolysis to erythritol	Data not available
Butylene Oxide	Merck 176	73.10	63	Alcohol, ether	Data not available
Epichlorhydrin	Merck 410	92.53	117.9	Ether Chloroform	Positive
Ethylene Dibromide	Merck 434	197.88	131-132	Alcohol = ROH Ether 250 parts H_2O	Weakly Positive
Ethylene Dichloride	Merck 434	98.96	83-84	120 parts H_2O ROH Ether	Positive
Ethylene Oxide	Merck 435	44.05	10.7-13.5	ROH H_2O Ether	Positive
Methyl Iodide	Merck 682	94.95	4.5	1.75 g/100 g soln ROH, ether	Data not available
Methylene Chloride	Merck 685	84.94	39.75	Soluble 50 parts H_2O, alcohol, ether dimethylformamide	Data not available
Methyl Iodide	Merck 688	141.9	42.5	Soluble 50 parts H_2O	Weakly Positive
Propylene Oxide	Merck 877	58.08	35	Alcohol, ether 100 parts H_2O	Data not available
Styrene Oxide	Merck 990	102.16	145	Alcohol, ether slightly in H_2O	Positive: solvent appears to be most important glycerol:H_2O:95% ethanol, optimal (2:2:1)
Vinyl Chloride	Merck 1108	62.50	-14	ROH	Weakly Positive
Vinylidene Chloride	Merck 1109	96.95	30-32	Insoluble H_2O	Weakly Positive

[a] Merck Index, Eight edition (1968).

[b] McCann et al. (1975).

[c] U. S. DHEW (1977).

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conducted in parallel with the mutagenicity experiments. Revertants/10^8 (surviving) cells were then calculated for each technique, which allowed comparison of mutagenic potency at each dose level. This calculation was performed to normalize both stationary- and log-phase cells to equal numbers of viable cells/dose level. This calculation (Revertants/10^8 cells) was then plotted on semi-log graph paper against dose level (µg/plate). Toxicity and mutagenicity data for each technique were plotted for the six compounds that were mutagenic (butylene oxide, propylene oxide, ethylene dibromide, styrene oxide, ethylene oxide, and vinylidene chloride - Figures 20 through 31). Graphs of raw data (*i.e.*, without calculation of revertants/survivor/10^8 cells) are also included for each positive compound; these graphs permit evaluation of the data without the bias of mathematical transformation.

The experimental results for each positive vapor-phase mutagenic compound with each of the three techniques follow.

Mutagenicity Testing of Butylene Oxide

The mutagenicity and toxicity raw data of butylene oxide with *Salmonella typhimurium* tester strain TA100 is plotted on Figure 20. Toxicity to TA100 was present in the preincubation technique with log-phase cells at 2,500 and 3,000 µg/plate. No toxicity was exhibited with either the log- or stationary-phase cells in the pour plate technique. Dose responsive mutagenicity in the pour plate technique with both log- and stationary-phase cells was not significant; however, mutagenicity in the preincubation technique demonstrated a linear dose response from 1,000-3,000 µg/plate. The mutagenic potential of butylene oxide with the preincubation technique was approximately eight fold greater than the spontaneous background rate and was 3-fold greater than the mutagenic potential in the pour plate technique with either log- or stationary-phase cells. These toxicity and mutagenicity results suggested that in the preincubation technique, the association of a volatile vapor-phase organic mutagen and the bacterial tester strain was optimized and was necessary for expression of mutagenic potential. When this raw data was transformed to normalize for toxicity and cell numbers in each technique (Figure 21 and Table 39), the mutagenicity of butylene oxide was again demonstrated. The preincubation technique was clearly superior in detecting the mutagenicity of butylene oxide. The transformed dose response curves for each technique were similar to graphs of the raw data (Figure 20). Consequently, the mathematical transformation of the raw data did not alter the conclusion about a compound's mutagenic potential. In addition, the extra histidine and biotin added in the preincubation technique did not increase the spontaneous background rate, and the standard deviation in the preincubation technique was lower than that in the pour plate technique with either log- or stationary-phase cells.

Mutagenicity Testing of Propylene Oxide

The raw toxicity and mutagenicity data of propylene oxide with *Salmonella* strain TA100 is contained in Figure 22. Propylene oxide was

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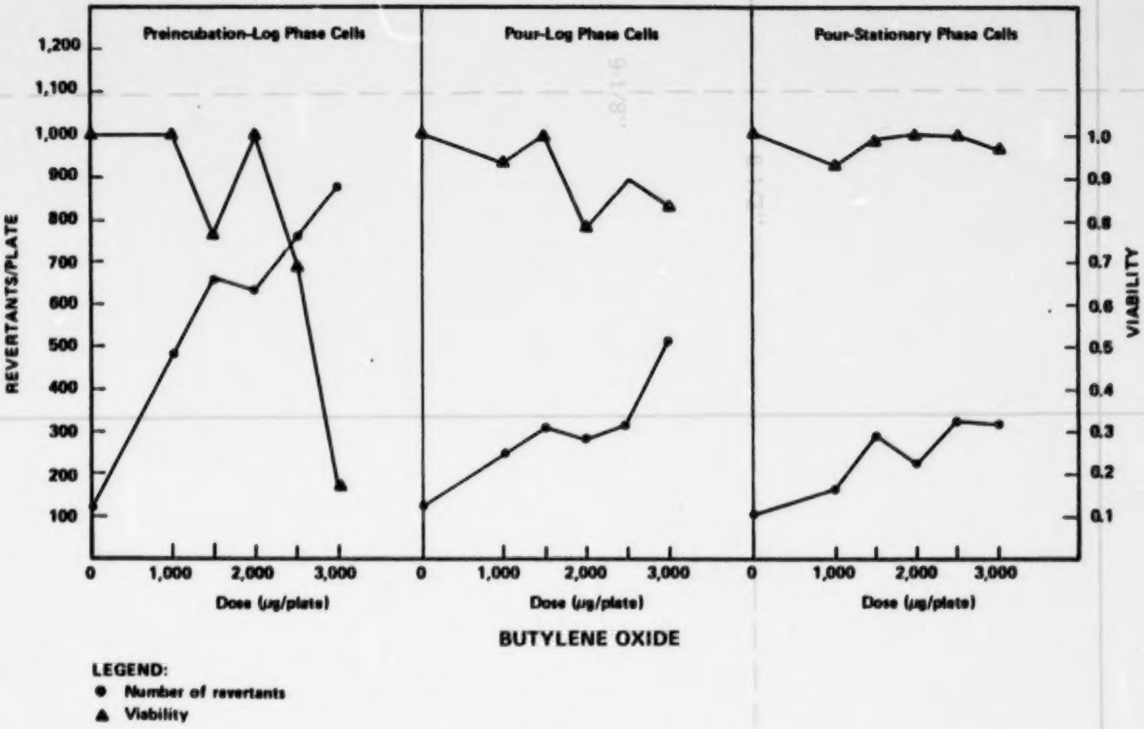

Figure 20. Raw mutagenicity and toxicity data for butylene oxide with Salmonella strain TA100 in three techniques.

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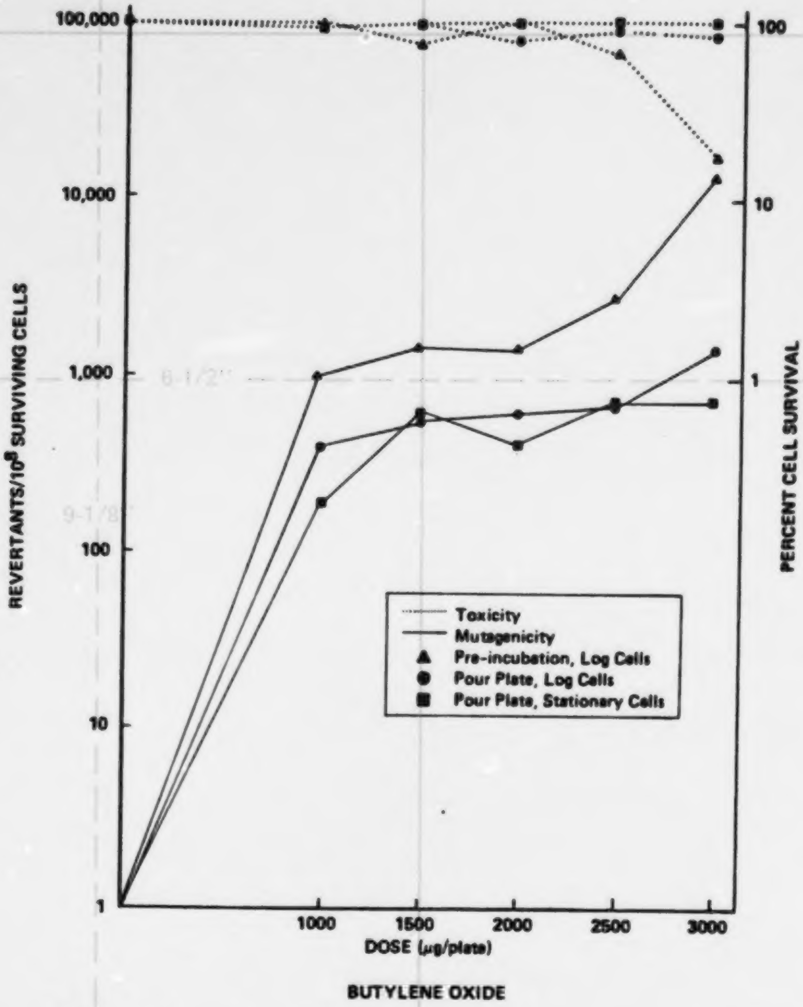

Figure 21. Transformed mutagenicity and toxicity data for butylene oxide with Salmonella strain TA100 in three techniques.

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TABLE 39. MUTAGENICITY AND TOXICITY DATA FOR BUTYLENE OXIDE WITH SALMONELLA STRAIN TA100 IN THREE TECHNIQUES

BUTYLENE OXIDE TA100

Dose μg/plate	TOTAL REVERTANTS			REVERTANTS-SBMR			REVERTANTS/SURVIVOR			REVERTANTS/10^8 CELLS		
	Pour Stationary	Pour Log	Preinc. Log	Pour Stationary	Pour Log	Preinc. Log	Pour Stationary	Pour Log	Preinc. Log	Pour Stationary	Pour Log	Preinc. Log
3000	318 ± 10.5	512 ± 78.8	881 ± 71.9	212	389	765	212	469	4500	718	1429	13719
2500	323 ± 80.2	316 ± 50.7	764 ± 16.2	217	193	648	217	217	939	735	661	2862
2000	219 ± 25.4	282 ± 56.3	634 ± 12.0	113	159	518	113	204	518	383	621	1579
1500	292 ± 69.2	308 ± 33.6	663 ± 35.6	186	185	547	186	185	547	630	564	1667
1000	163 ± 15.5	251 ± 81.4	491 ± 12.5	57	128	375	57	128	375	193	390	1143
SBMR	106 ± 13.0	123 ± 20.8	116 ± 31.5									

EXPERIMENTAL CONDITIONS

Stationary	Log	
369	493	CFU Control
1/80000	1/66600	Dilution
2.95×10^7	3.28×10^7	Cells Plated
.295	.328	Cells 10^8

CFU: Colony Forming units (bacteria)
Preinc.: Preincubation 37°C 30 min
Log: Cells used in logarithmic growth phase
SBMR: Spontaneous back-mutation rate

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not significantly toxic to the bacteria under the conditions tested. A gradual increase in mutagenic sensitivity was detected from the low response demonstrated in the plate incorporation technique with stationary-phase cells to the high response demonstrated in the preincubation technique with log-phase cells. A linear dose response was demonstrated for propylene oxide with preincubation and log-phase cells from 250 to 5,000 µg/plate. Propylene oxide was more mutagenic in the preincubation technique (with log-phase cells) than in the pour plate technique (with log-phase cells). In addition, log-phase cells were superior to stationary-phase cells in detecting the mutagenicity of propylene oxide. These results were more clearly demonstrated when this data was plotted to normalize for cell number in each technique (Figure 23 and Table 40). Little or no mutagenic potential was exhibited by this epoxide with the standard Ames plate incorporation technique and stationary-phase cells. The preincubation technique with log-phase cells was the most sensitive method of detection of propylene oxide at all dose levels tested.

Mutagenicity Testing of Ethylene Dibromide

The mutagenicity of ethlyene dibromide demonstrated a linear dose response in the preincubation technique with log-phase cells from 50-500 µg/plate with *Salmonella* strain TA100. The mutagenic potential was 2-3X greater in the preincubation technique. A much smaller mutagenic potential was demonstrated in the plate incorporation technique with either log- or stationary-phase cells. This mutagenic potential was demonstrated in both the raw data and transformed data graphs (Figures 24 and 25, respectively). The standard deviation was smallest in the preincubation technique (Table 41). No significant toxicity was demonstrated in any of the three techniques.

Mutagenicity Testing of Styrene Oxide

The toxicity and mutagenicity data of the volatile vapor-phase organic mutagen styrene oxide with *Salmonella* strain TA100 are plotted on Figures 26 and 27, and the raw data is tabulated in Table 42. Toxicity was significant with all three techniques at doses above 250 µg/plate; styrene oxide was the most toxic in the preincubation assay. Mutagenic potential was greater in the preincubation technique with log-phase cells and a linear dose response was evident from 100-500 µg/plate. Higher toxicity in the preincubation technique caused the mutagenic potential at higher doses (500 and 1,000 µg/plate) to be slightly depressed when compared to the pour plate technique with log-phase cells. Mutagenic potential was demonstrated in the pour plate technique with log-phase cells from 100-750 µg/plate, but a lower mutagenic potential was demonstrated with stationary-phase cells. The raw data (Table 42) indicated a mutagenic ratio of between 4.0 and 5.0 in both the pour plate and preincubation technique with log-phase cells at dose levels of 1,000 and 750 µg/plate. A mutagenic ratio of approximately 3.0 was indicated in the pour plate assay with stationary phase cells at 1,000 µg of styrene oxide/plate. However, excessive toxicity made interpretation of data points beyond 500 µg/plate difficult, especially in the preincubation assay.

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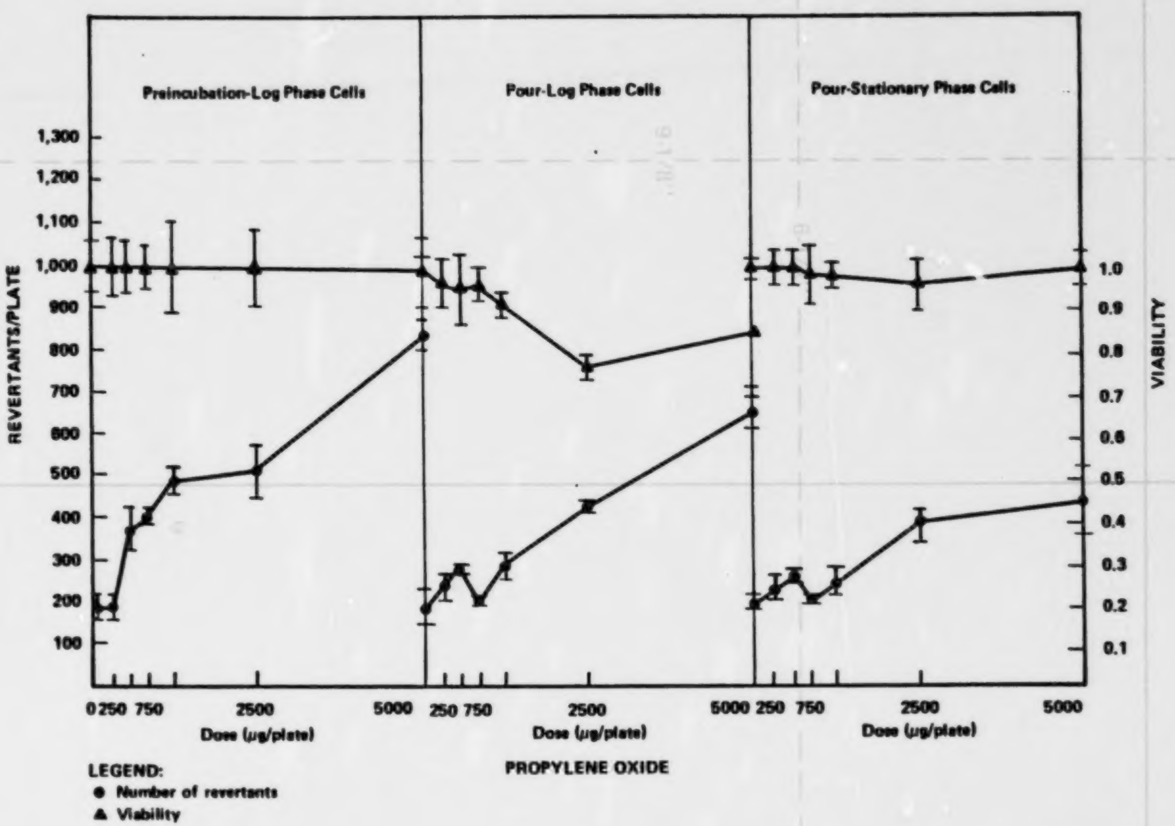

Figure 22. Raw mutagenicity and toxicity data for propylene oxide with Salmonella strain TA100 in three techniques.

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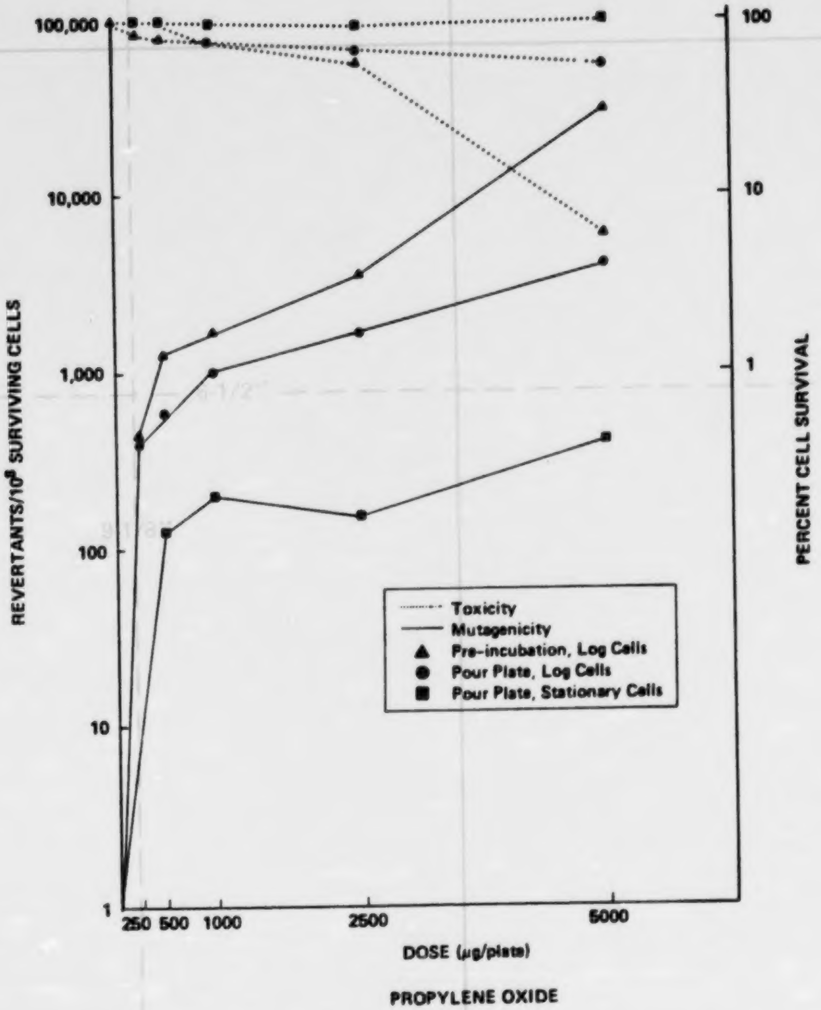

Figure 23. Transformed mutagenicity and toxicity data for propylene oxide with _Salmonella_ strain TA100 in three techniques

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TABLE 40. MUTAGENICITY AND TOXICITY DATA FOR PROPYLENE OXIDE WITH SALMONELLA STRAIN TA100 IN THREE TECHNIQUES

PROPYLENE OXIDE TA100

Dose µg/plate	TOTAL REVERTANTS Pour Stationary	TOTAL REVERTANTS Pour Log	TOTAL REVERTANTS Preinc. Log	REVERTANTS-SBMR Pour Stationary	REVERTANTS-SBMR Pour Log	REVERTANTS-SBMR Preinc. Log	REVERTANTS/SURVIVOR Pour Stationary	REVERTANTS/SURVIVOR Pour Log	REVERTANTS/SURVIVOR Preinc. Log	REVERTANTS/10^8 CELLS Pour Stationary	REVERTANTS/10^8 CELLS Pour Log	REVERTANTS/10^8 CELLS Preinc. Log
5000	336 ± 2.1	862 ± 74.2	822 ± 113.4	160	678	645	160	1356	10750	416	4023	31899
2500	238 ± 11.0	634 ± 73.7	862 ± 66.4	62	450	685	62	570	1181	161	1691	3504
1000	255 ± 7.2	464 ± 13.0	626 ± 39.8	79	280	449	79	329	583	205	976	1729
750	205 ± 25.5	329 ± 36.3	459 ± 27.6	29	145	282	29	145	344	75	430	1020
500	228 ± 30.6	389 ± 43.4	555 ± 30.3	52	205	378	52	205	445	135	608	1320
250	160 ± 20.8	318 ± 16.5	328 ± 43.4	-16	134	151	-16	134	151	-	397	448
SBMR	176 ± 33.7	184 ± 12.2	177 ± 15.4									

EXPERIMENTAL CONDITIONS

Stationary	Log	
480.5	505.6	CFU Control
1/80000	1/66600	Dilution
3.84×10^7	3.37×10^7	Cells Plated
.384	.337	Cells 10^8

CFU: Colony Forming units (bacteria)
Preinc.: Preincubation 37°C 30 min
Log: Cells used in logarithmic growth phase
SBMR: Spontaneous back-mutation rate

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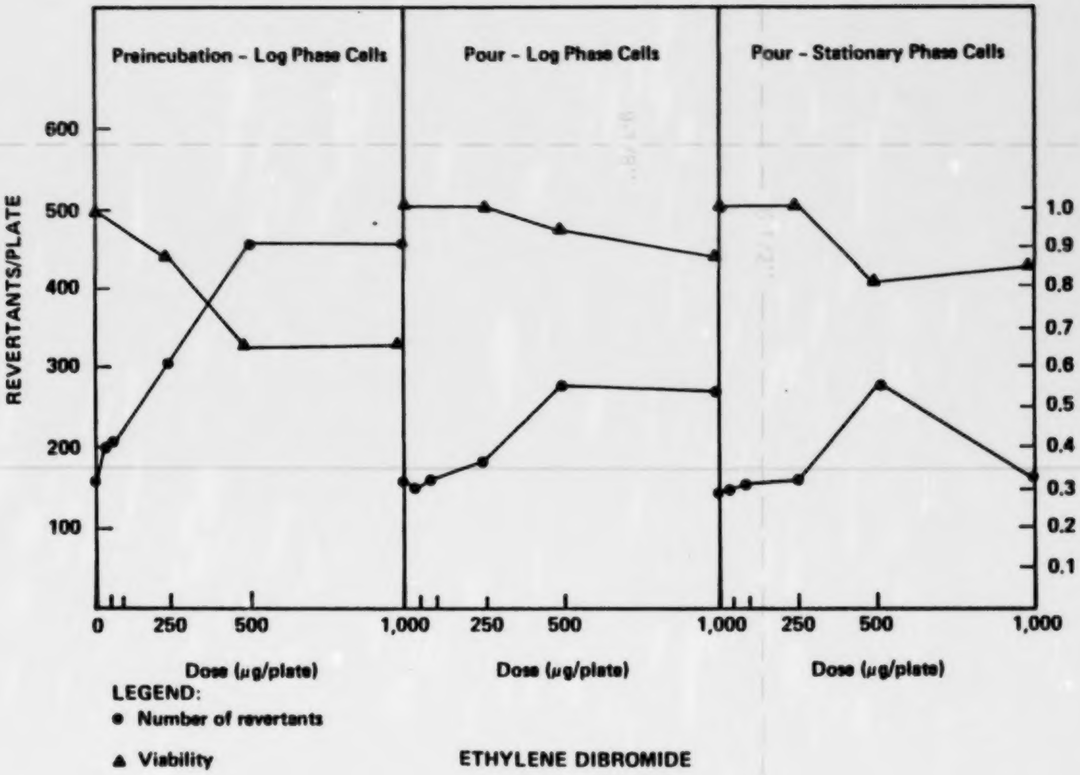

Figure 24. Raw mutagenicity and toxicity data for ethylene dibromide with *Salmonella* strain TA100 in three techniques.

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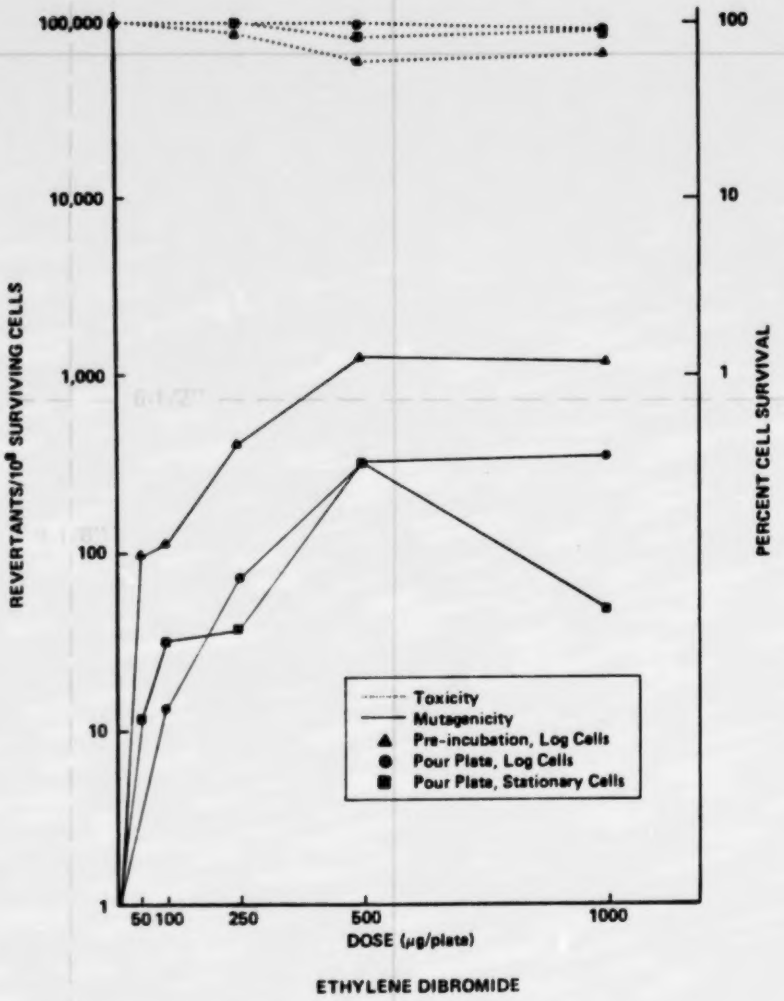

Figure 25. Transformed mutagenicity and toxicity data for ethylene dibromide with Salmonella strain TA100 in three techniques.

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TABLE 41. MUTAGENICITY AND TOXICITY DATA FOR ETHYLENE DIBROMIDE WITH SALMONELLA STRAIN TA100 IN THREE TECHNIQUES

ETHYLENE DIBROMIDE TA100

Dose μg/plate	TOTAL REVERTANTS			REVERTANTS-SBMR			REVERTANTS/SURVIVOR			REVERTANTS/10^8 CELLS		
	Pour Stationary	Pour Log	Preinc. Log	Pour Stationary	Pour Log	Preinc. Log	Pour Stationary	Pour Log	Preinc. Log	Pour Stationary	Pour Log	Preinc. Log
1000	160 ± 35.0	263 ± 4.9	448 ± 20.5	17	106	283	20	122	429	47	383	1191
500	283 ± 37.0	275 ± 23.6	456 ± 12.7	140	118	291	139	118	485	327	327	1347
250	159 ± 20.9	193 ± 20.8	304 ± 4.9	16	26	139	16	26	158	37	72	438
100	157 ± 12.3	162 ± 2.9	206 ± 2.1	14	5	41	14	5	41	33	13	113
50	148 ± 12.1	149 ± 12.2	200 ± 1.4	5	-	35	5	-	35	11	-	97
SBMR	143 ± 26.2	157 ± 10.3	165 ± 1.4									

EXPERIMENTAL CONDITIONS

Stationary	Log	
530	541	CFU Control
1/80000	1/66600	Dilution
4.24×10^7	3.60×10^7	Cells Plated
.424	.360	Cells 10^8

CFU: Colony forming units (bacteria)
Preinc.: Preincubation 37°C 30 min
Log: Cells used in logarithmic growth phase
SBMR: Spontaneous back-mutation rate

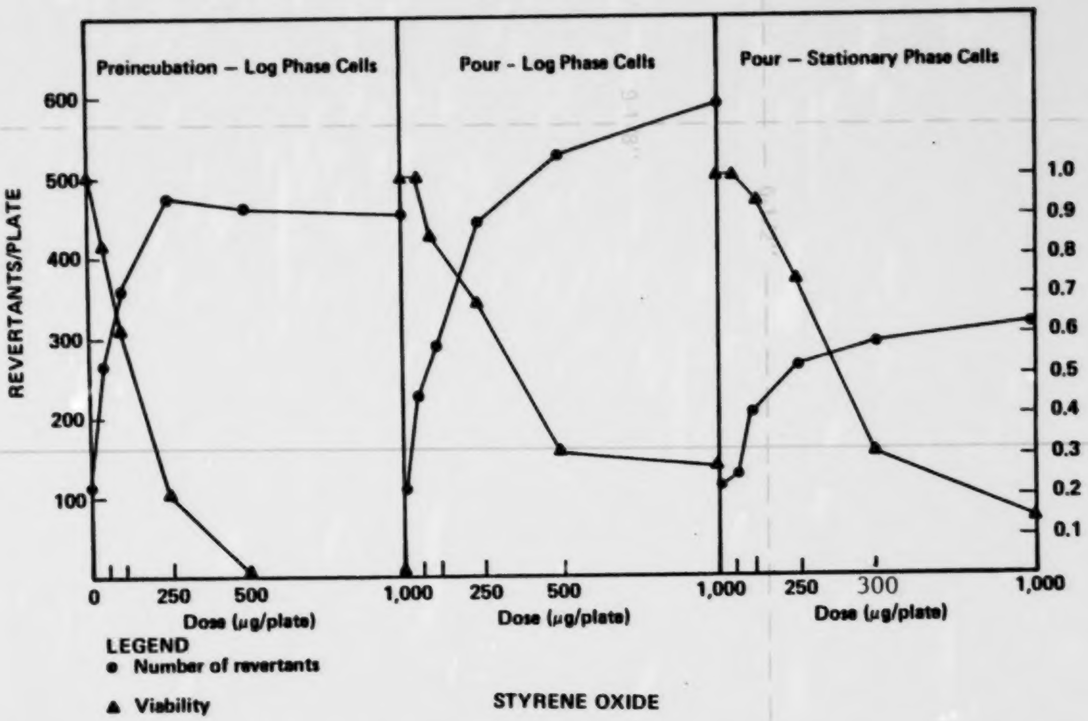

Figure 26. Raw mutagenicity and toxicity data for styrene oxide with Salmonella strain TA100 in three techniques.

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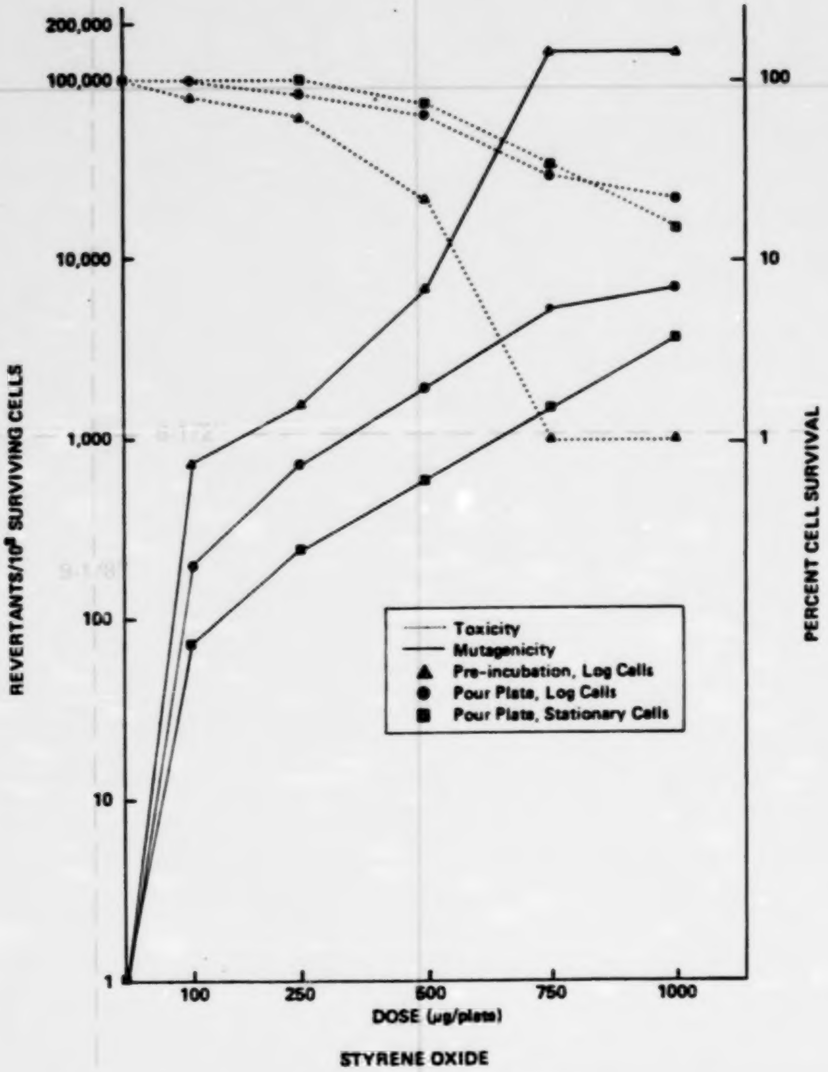

Figure 27. Transformed mutagenicity and toxicity data for styrene oxide with Salmonella strain TA100 in three techniques.

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TABLE 42. MUTAGENICITY AND TOXICITY DATA FOR STYRENE OXIDE WITH SALMONELLA STRAIN TA100 IN THREE TECHNIQUES

STYRENE OXIDE TA100

Dose µg/plate	TOTAL REVERTANTS Pour Stationary	Pour Log	Preinc. Log	REVERTANTS-SBMR Pour Stationary	Pour Log	Preinc. Log	REVERTANTS/SURVIVOR Pour Stationary	Pour Log	Preinc. Log	REVERTANTS/10^8 CELLS Pour Stationary	Pour Log	Preinc. Log
1000	318 ± 61.5	584 ± 72.7	451 ± 41.9	208	451	331	1387	1670	33100	3863	7136	141452
750	292 ± 39.9	528 ± 40.3	463 ± 48.1	182	395	343	569	1274	34300	1584	5444	146581
500	266 ± 21.7	445 ± 41.6	473 ± 26.9	156	312	353	211	459	1681	587	1961	7183
250	203 ± 3.1	285 ± 24.0	355 ± 11.2	93	152	235	93	179	379	259	764	1619
100	137 ± 8.3	179 ± 11.8	264 ± 17.3	27	46	144	27	46	173	75	196	739
SBMR	110 ± 7.2	133 ± 9.1	120 ± 2.1									

EXPERIMENTAL CONDITIONS

Stationary	Log	
489	351	CFU Control
1/80000	1/66600	Dilution
3.59×10^7	2.34×10^7	Cells Plated
.359	.234	Cells 10^8

CFU: Colony forming units (bacteria)
Preinc.: Preincubation 37°C 30 min
Log: Cells used in logarithmic growth phase
SBMR: Spontaneous back-mutation rate

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Mutagenicity Testing of Ethylene Oxide

The raw mutagenicity results of ethylene oxide in all three techniques tested are plotted in Figure 28. A positive response with preincubation and log-phase cells was suggested by this data, since all dose levels demonstrated an increasing linear mutagenic response. No toxicity was present with any technique. The mutagenicity results of ethylene oxide in the plate incorporation and preincubation techniques with log-phase cells were similar except for the highest dose of 2,000 μg/plate (Figure 29 and Table 43). Log-phase cells did significantly increase the mutagenic response of ethylene oxide to TA100 when the revertants per 10^8 cells was calculated (Figure 29 and Table 43). The enhancement of activity with log-phase versus stationary-phase cells in the plate incorporation assay was not apparent when the raw data was graphed (Figure 28), but the difference became apparent when the data was calculated to account for cell number (Figure 27).

Mutagenicity Testing of Vinylidene Chloride

The interpretation of the mutagenicity data for vinylidene chloride with TA100 was difficult, due to the high toxicity present in the preincubation assay with log-phase cells, and by the high spontaneous background rate with all three techniques (Figures 30, 31, and Table 44). The data for vinylidene chloride are included in this report because vinylidine chloride was the only compound that was tested with S9 addition. It was known from previous studies that vinylidene chloride required S9 addition for activity (Malaveille et al., 1975, 1977). The strategy utilized in this pilot study was to first test all compounds without S9 addition, then continue the study with S9 addition. Vinylidene chloride was tested with S9 initially because it was known to require S9 activation. The high spontaneous background rate was probably due to an excessive amount of S9 protein added per plate (6.0 mg of S9 protein/plate), and a 72 hr incubation period.

Mutagenic ratios between 3.0 and 4.0 were present in the pour and preincubation technique with log-phase cells at dose levels of 1,000, 2,000, 2,500, and 3,000 μg/plate. However, the spontaneous background rate with S9 addition averaged 320 revertant colonies/plate. The mutagenic ratios suggested that mutagenic potential did exist for vinylidene chloride and was not caused by the high background spontaneous rate since the total number of revertants was large (>1,200) at the highest dose (Table 44). The number of revertants with log-phase cells in both the pour plate and preincubation techniques were greater than those exhibited by stationary-phase cells in the standard plate incorporation technique. Significant toxicity was present only in the preincubation technique and was present at dose levels above 2,000 μg/plate. Vinylidene chloride should be further studied with this testing protocol by varying the S9 concentration per plate and the inducer of S9 activity. In addition, the use of an NADPH generating system (Ames et al., 1975) instead of the use of NADPH directly, may increase the mutagenic potential of vinylidene chloride, and the detection ability of promutagens in general.

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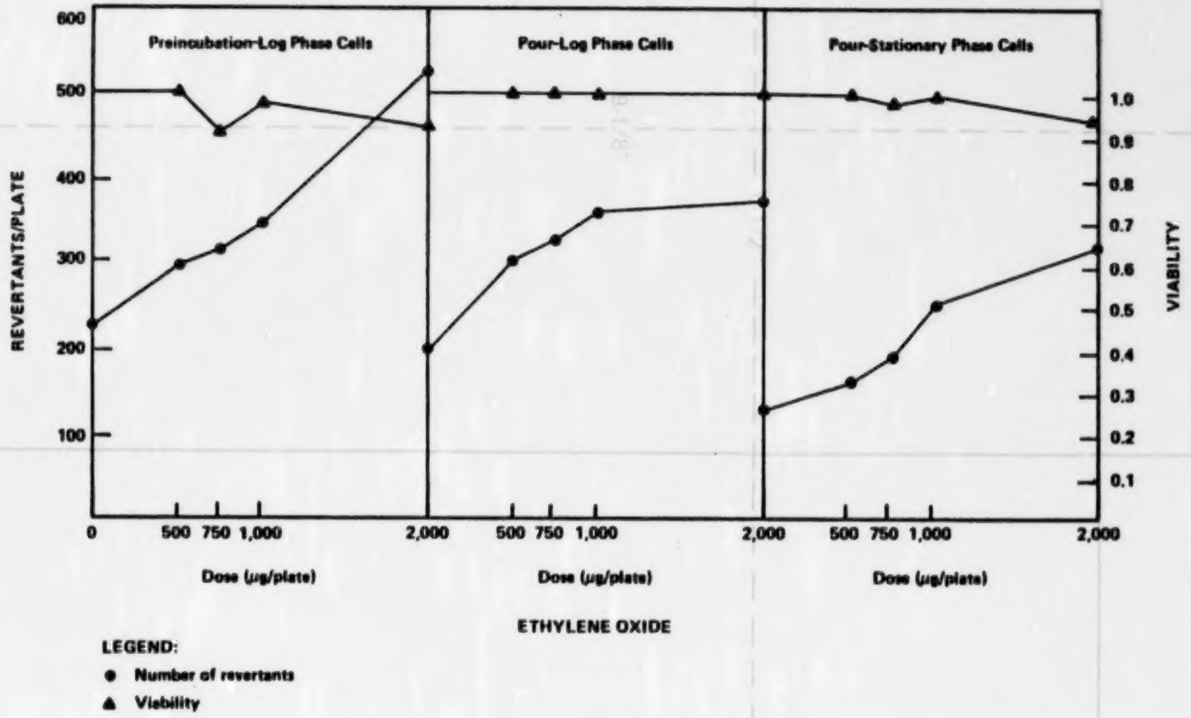

Figure 28. Raw mutagenicity and toxicity data for ethylene oxide with Salmonella strain TA100 in three techniques.

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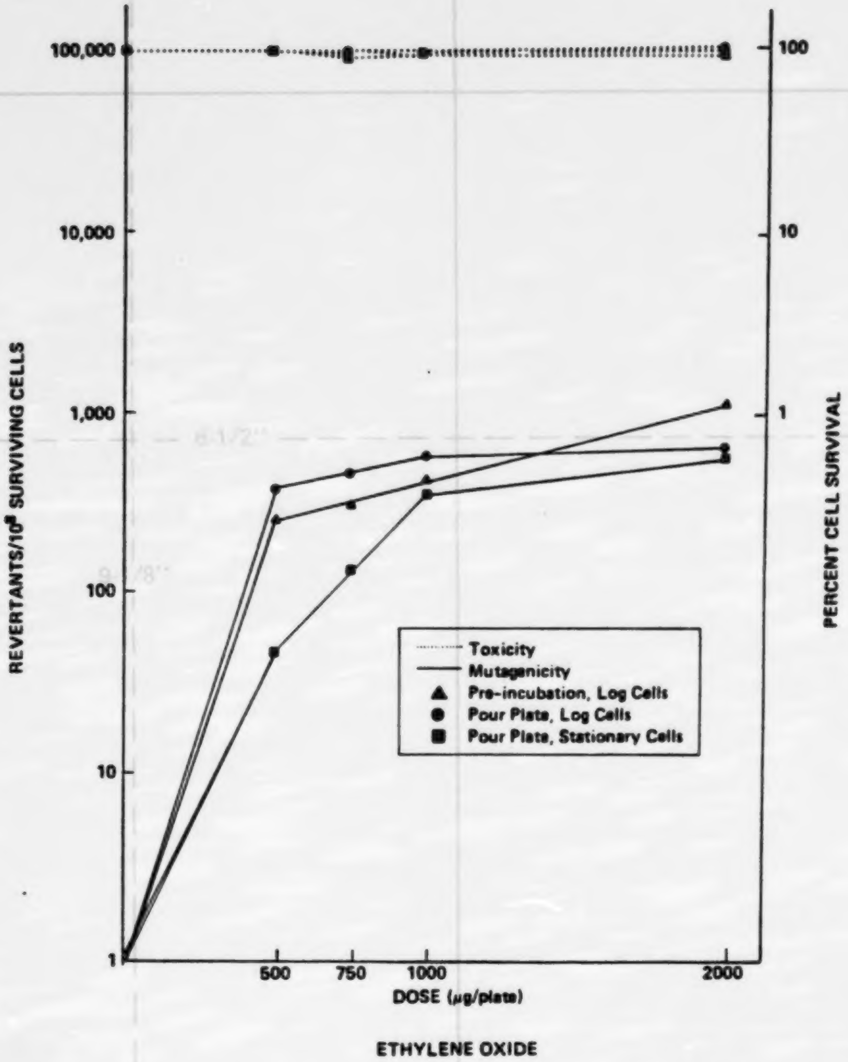

Figure 29. Transformed mutagenicity and toxicity data for ethylene oxide with Salmonella strain TA100 in three techniques.

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TABLE 43. MUTAGENICITY AND TOXICITY DATA FOR ETHYLENE OXIDE WITH SALMONELLA STRAIN TA100 IN THREE TECHNIQUES

ETHYLENE OXIDE TA100

Dose µg/plate	TOTAL REVERTANTS			REVERTANTS-SBMR			REVERTANTS/SURVIVOR			REVERTANTS/10^8 CELLS		
	Pour Stationary	Pour Log	Preinc. Log	Pour Stationary	Pour Log	Preinc. Log	Pour Stationary	Pour Log	Preinc. Log	Pour Stationary	Pour Log	Preinc. Log
2000	325 ± 26.6	373 ± 15.5	526 ± 67.9	177	166	293	177	166	293	596	628	1109
1000	255 ± 22.3	359 ± 16.2	348 ± 21.7	107	152	115	107	152	115	344	575	435
750	191 ± 23.2	331 ± 18.3	315 ± 11.5	43	124	82	43	124	82	138	469	310
500	162 ± 2.1	304 ± 27.9	299 ± 30.6	14	97	66	14	97	66	45	367	250
SBMR	148 ± 19.1	207 ± 70.4	233 ± 30.8									

EXPERIMENTAL CONDITIONS

Stationary	Log	
389	396	CFU Control
1/80000	1/66600	Dilution
3.11×10^7	2.64×10^7	Cells Plated
.311	.264	Cells 10^8

CFU: Colony Forming units (bacteria)
Preinc.: Preincubation 37°C 30 min
Log: Cells used in logarithmic growth phase
SBMR: Spontaneous back-mutation rate

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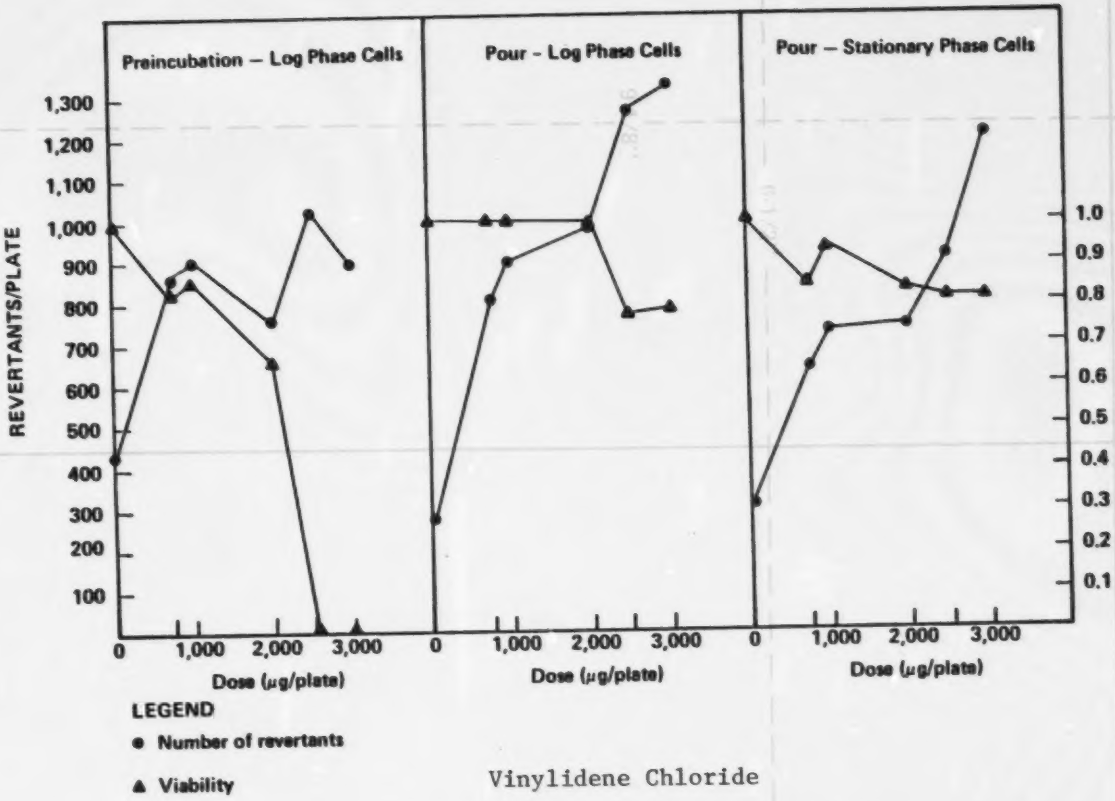

Vinylidene Chloride

Figure 30. Raw mutagenicity and toxicity data for vinylidene chloride with Salmonella strain TA100 in three techniques.

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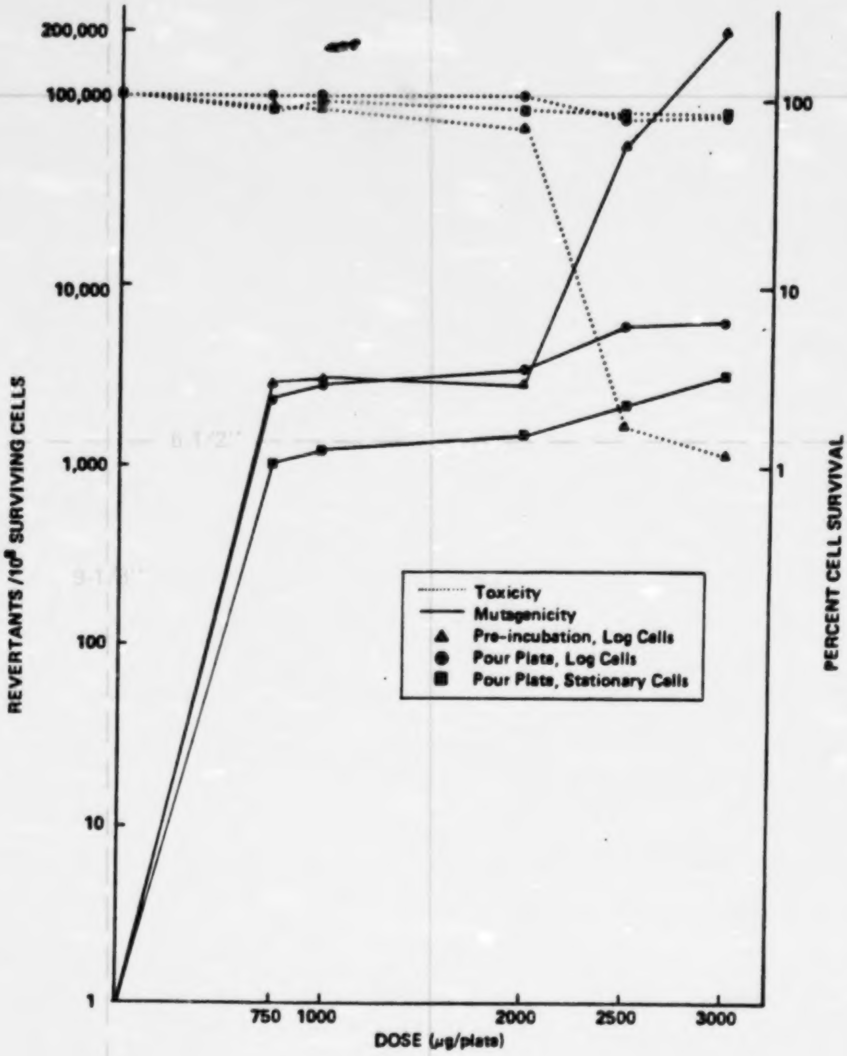

Vinylidene Chloride

Figure 31. Transformed mutagenicity and toxicity data for vinylidine chloride with Salmonella strain TA100 in three techniques.

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TABLE 44. MUTAGENICITY AND TOXICITY DATA FOR VINYLIDINE CHLORIDE WITH SALMONELLA STRAIN TA100 IN THREE TECHNIQUES

Vinylidene Chloride TA100

Dose µg/plate	TOTAL REVERTANTS Pour Stationary	Pour Log	Preinc. Log	REVERTANTS-SBMR Pour Stationary	Pour Log	Preinc. Log	REVERTANTS/SURVIVOR Pour Stationary	Pour Log	Preinc. Log	REVERTANTS/10^8 CELLS Pour Stationary	Pour Log	Preinc. Log
3000	1208 ± 120.0	1317 ± 30.7	898 ± 138.4	903	1009	531	1115	1293	53100	2896	5671	232895
2500	910 ± 96.9	1269 ± 31.0	1028 ± 143.0	605	961	661	747	1248	13220	1940	5474	57982
2000	739 ± 58.9	978 ± 41.2	758 ± 58.6	434	670	391	523	670	592	1358	2939	2596
1000	727 ± 20.3	902 ± 6.4	903 ± 55.8	422	594	536	454	594	623	1179	2605	2737
750	618 ± 64.0	807 ± 81.0	870 ± 36.1	313	499	503	373	499	613	969	2189	2689
SBMR	309 ± 20.0	308 ± 12.0	367 ± 29.4									

EXPERIMENTAL CONDITIONS

Stationary	Log	
481	341.75	CFU Control
1/80000	1/66600	Dilution
3.85×10^7	2.28×10^7	Cells Plated
.385	.228	Cells 10^8

CFU: Colony forming units (bacteria)
Preinc.: Preincubation 37°C 30 min
Log: Cells used in logarithmic growth phase
SBMR: Spontaneous back-mutation rate

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CHEMICAL IDENTIFICATION OF MUTAGENIC AND/OR CARCINOGENIC COMPOUNDS IN POM AND VPOs FROM SEVEN GEOGRAPHICAL LOCATIONS

Vapor-phase organics (VPOs) and particulate organic matter (POM) were chemically analyzed with the GC/MS/Computer system (described in Materials and Methods - Section 4). Table 45 lists the mutagenic and/or carcinogenic signature compounds identified from seven locations: South Charleston, West Virginia; Baton Rouge, Louisiana; Lake Charles, Louisiana; Beaumont, Texas; Houston, Texas; Upland, California; and Elizabeth, New Jersey. Limited sample size did not permit the chemical analysis of all particulate chemical-class fractions from all sites, and only selected sites were chosen for vapor-phase organic analysis. Qualitative chemical analysis indicated that: (1) the PNA fraction contained the greatest number of mutagenic/carcinogenic compounds, which correlated with the mutagenic potential of the PNA fraction; (2) spillover of PNA compounds into the PN fraction was detected; (3) compounds identified included both direct-acting mutagens (e.g., 2-nitro-4,6-dichlorophenol from South Charleston) and promutagens (e.g., benzo-a-pyrene); (4) the basic and acidic fractions contained fewer positive mutagenic and carcinogenic compounds than did the PNA fraction; (5) the NPN fractions contained only a few mutagenic/carcinogen compounds; this finding correlated with the mutagenic potential of this fraction; (6) significant chemical spillover of PNA compounds into the PN fraction of the South Charleston, West Virginia) sample was detected. This high spillover of PNA compounds resulted from the initial fractionation scheme; spillover was reduced by the final fractionation scheme with the other six samples tested; and (7) the majority of vapor-phase organic mutagenic and/or carcinogenic compounds identified were short-chained chlorinated hydrocarbons (e.g., tetrachloroethylene).

TABLE 45. MUTAGENIC AND/OR CARCINOGENIC COMPOUNDS[a] IN AMBIENT PARTICULATE ORGANIC MATTER (POM) AND VAPOR-PHASE (VPO) FROM SEVERAL LOCATIONS; IDENTIFICATION WAS PERFORMED BY THE GC/MS/COMPUTER SYSTEM

	PNA	PN	ACIDS	BASES	NPN	VAPORS
South Charleston, West Virginia[b] I (<1.7 µm)	benzanthrene[m,c] benzfluoranthene[c] benzopyrene[m,c] chrysene[m,c] fluoranthene[c] perylene[c]	cresol[m,c] phenol[c]	phenol[c]	quinoline[m] nicotine[m,c] 2-nitro-4,6-dichlorophenol[m,c]	cresol[m,c] phenol[c]	NOT TESTED NO SAMPLE AVAILABLE
South Charleston, West Virginia[b] II (<1.7 µm)	anthracene[c] methylnaphthalene[c] naphthalene[c]	acridine[m,c] benzofluoroanthene[c] benzopyrene[m,c] chrysene[m,c] fluoranthene[c] methylbenzanthracene[c] methylbenzophenanthracene[c] naphthalene[c]	quinoline[c]	quinoline[m] naphthalene[c] nicotine[m,c] phenol[c]	benzoquinoline[c]	NOT TESTED NO SAMPLE AVAILABLE

(continued)

TABLE 45 (cont'd)

	PNA	PN	ACIDS	BASES	NPN	VAPORS
Baton Rouge, Louisiana (<1.7 μm)	anthracene^{c} benzopyrene^{m,c} fluoranthene^{c} phenanthrene^{c} pyrene^{c}	NO POSITIVE COMPOUNDS IDENTIFIED	cresol^{m,c} phenol^{c}	quinoline^{m}	NO POSITIVE COMPOUNDS IDENTIFIED	NOT TESTED NO SAMPLE AVAILABLE
Baton Rouge, Louisiana (>1.7 μm)	anthracene^{c} benzanthracene^{m,c} benzfluoranthene^{c} benzo(a)pyrene^{m,c} fluoranthene^{c} methylbenzanthracene^{m,c} methylphenanthrene^{c} naphthalene^{c} phenanthrene^{c} pyrene^{c}	benzo(a)pyrene^{m,c} benzfluoranthene^{c}	cresol^{m,c} phenol^{c}	benzoacridine^{c} benzoquinoline^{c} quinoline^{m} nicotine^{m,c}	NO POSITIVE COMPOUNDS IDENTIFIED 87 SATURATED AND UNSATURATED HYDROCARBONS WERE IDENTIFIED HOWEVER	

(continued)

TABLE 45 (cont'd)

	PNA	PM	ACIDS	BASES	NPM	VAPORS
Lake Charles, Louisiana (<1.7 µm	anthracene^{c}	acridine^{m,c}	cresol^{m,c}	benzoquinoline^{c}	NOT TESTED NO SAMPLE AVAILABLE	benzene^{c}
	anthraquinone^{c}	aniline^{c}	naphthacene^{c}	dibenzylamine^{c}		carbon tetrachloride^{c}
	benzanthracene^{m,c}	benzene^{c}	naphthalene^{c}	dicyclohexyl-amine^{c}		chloroform^{c}
	benzfluoranthene^{c}	benznanthracene^{m,c}	phenol^{c}	nicotine^{m,c}		naphthalene^{c}
	benzperylene^{c}	benzophenanthrene^{c}	phenyl-phenyl^{c}			trichloro-ethylene^{m,c}
	benzophenanthrene^{c}	benzoquinoline^{c}	p-tetramethyl-butylphenol^{c}			vinyl chloride^{m,c}
	benzopyrene^{c}	chrysene^{m,c}				vinylidene chloride^{m,c}
	chrysene^{c}					
	fluorathene^{c}					
	methylbenzanthracene^{c}					
	methylchrysene^{c}					
	methylbenzophenanthracene^{c}					
	methylphenanthrene^{c}					
	methylpyrene^{c}					
	methylstearate^{c}					
	perylene^{c}					
	phenanthrene^{c}					
	pyrene^{c}					

(continued)

TABLE 45 (cont'd)

	PNA	PN	ACIDS	BASES	NPN	VAPORS
Beaumont,	NOT TESTED NO SAMPLE AVAILABLE					benzene[c] (and benzene isomers) carbon tetrachloride[c] chloroform[m,c] naphthalene[c] phenol[c] tetrachloroethylene[m,c]

(continued)

TABLE 45 (cont'd)

	PNA	PN	ACIDS	BASES	NPN	VAPORS
Houston, Texas (2566-1,2) <1.7 µm	benzopyrene^{m,c} pyrene^{c}	NO POSITIVE COMPOUNDS IDENTIFIED	cresol^{m,c} naphthalene^{c} phenol^{c}	NOT TESTED NO SAMPLE AVAILABLE	NOT TESTED NO SAMPLE AVAILABLE	benzene^{c} carbon tetrachloride^{c} ethylenedibromide^{c} trichloroethane^{c} tetrachloroethylene^{m,c} trichloroethylene^{m,c}
Houston, Texas (2566-1,2) >1.7 µm	benzofluoranthene^{c} benzopyrene^{m,c} chrysene^{m,c}	NOT TESTED NO SAMPLE AVAILABLE	benzene^{c} isomers phenol^{c} isomers	NOT TESTED NOT SAMPLE AVAILABLE	NOT TESTED NO SAMPLE AVAILABLE	

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TABLE 45 (cont'd)

	PNA	PN	ACIDS	BASES	HPN	VAPORS
Upland, California (2566-53,54) <1.7 μm	NOT TESTED	fluoranthene^c phenol^c pyrene^c	cresol^c phenol^c	NOT TESTED	NOT TESTED	NOT TESTED
Upland, California (2566-55,56 >3.5 μm	anthracene^c benzathracene^c benzophenanthrene^c fluoranthene^c methylphenanthrene^c naphthacene^c perylene^c phenanthrene^c pyrene^c	NO POSITIVE COMPOUNDS IDENTIFIED	NO POSITIVE COMPOUNDS IDENTIFIED	NOT TESTED	NOT TESTED	

(continued)

TABLE 45 (cont'd)

	PNA	PN	ACIDS	BASES	NPN	VAPORS
Upland, California (1.7-3.5 µm)	anthracene[c] benzanthracene[c] benzophenanthrene[c] fluoranthene[c] methylphenanthrene[c] naphthacene[c] phenanthrene[c] perylene[c] pyrene[c]	NO POSITIVE COMPOUNDS IDENTIFIED	naphthalene[c]	NOT TESTED	NOT TESTED	NOT TESTED

(continued)

TABLE 45 (cont'd)

	PNA	PN	ACIDS	BASES	NPN	VAPORS
Elizabeth, New Jersey (2566-60,61) <1.7 µm	anthracene^{c} benzathracene^{m,c} benzofluoranthene^{m,c} benzophenanthrene^{m,c} benzopyrene^{m,c} chrysene^{m,c} methylbenzanthracene^{m,c} methylbenzophenanthrene^{c} methylchrysene^{c} phenanthracene^{c} phenylnapthylamine^{m,c}	fluoranthene^{c}	NO POSITIVE COMPOUNDS IDENTIFIED	NOT TESTED	NOT TESTED	VEZ-6 benzene^{c} (and benzene isomers) carbon tetrachloride^{c} chloroform^{c} naphthalene^{c} phenol^{c} tetrachloroethylene^{m,c}
Elizabeth, New Jersey (2566-62,63) 1.7-3.5 µm	anthracene^{c} benzoanthracene^{m,c} benzopyrene^{m,c} benzofluoranthene^{c} benzophenanthrene^{c} chrysene^{m,c} phenanthrene^{c}	benzophenanthrene^{c} chrysene^{m,c}	NO POSITIVE COMPOUNDS IDENTIFIED	NOT TESTED	NOT TESTED	

(continued)

TABLE 45 (cont'd)

	PNA	PN	ACIDS	BASES	NPN	VAPORS
						VEZ-10
Elizabeth, New Jersey (2566-64,65) >3.5 µm	anthracene[c] benzopyrene[m,c] benzofluoranthene[c] benzophenanthrene[m,c] chrysene[m,c]	NO POSITIVE COMPOUNDS IDENTIFIED	NOT TESTED	NOT TESTED	NOT TESTED	benzene[c] (and benzene isomers) carbon tetrachloride[c] chloroform[c] naphthalene[c] phenol[c] trichloroethylene[m,c] tetrachloroethylene[m,c]

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[a] Carcinogen and mutagen designations from: McCann et al., 1975; Sawicki, 1979; Rinkus and Legator, 1979.

[b] These samples were fractionated by the 1st fractionation scheme; all other samples were fractionated by the 2nd fractionation scheme. Chemical spillover was high with the 1st scheme, as indicated by the numerous PNA compounds identified in the PN class. This spillover was reduced with the 2nd (more simple) fractionation scheme.

[c] Carcinogen.

[m] Mutagen.

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SECTION 6

DISCUSSION

The discussion is divided into three parts: (1) the chemical and biological characterization of particulate organic matter; (2) the development of techniques to assess the mutagenicity of vapor-phase organics; and (3) the protocol developed for minimal biological and chemical analysis of ambient air samples. This discussion does not review the specifics of the data already presented in the Materials and Methods (Section 4) and the Results (Section 5). Rather, results are summarized with respect to the biological, chemical, and physical correlations and to procedures for adequate evaluation of the mutagenic potential of ambient air.

PARTICULATE ORGANIC MATTER (POM)

The chemical and biological characterization of atmospheric pollutants can determine their primary sources, their chemical transformation pathways in the atmosphere, and their potential risk to human health. Such determinations provide guidelines for the development of reasonable control strategies. Toxic compounds present in the atmosphere include both vapor-phase organics (VPO) (NRC, 1976) and particulate organic matter (POM) (NRC, 1979); it is therefore important that sample collection and analytical techniques encompass the entire spectrum of substances.

Air pollutants can be classified as either primary or secondary. Primary atmospheric pollutants are natural (e.g., dusts, vegetation, etc.) or anthropogenic in origin [e.g., smoke stack and vehicle emissions (Fishbein, 1976)]. Secondary atmospheric pollutants are generated from primary pollutants via atmospheric (e.g., photochemical) reactions. According to Hidy and Brock (1970), the majority of the global particulate load is of natural origin (~8 x 10^{11} g/day from dust, sea spray, forest fires). The production rate from human activities is on the order of 5.6 x 10^{11} g/day, mostly from combustion and industrial processes. The concentration range of aerosol in air extends from ~100-200 μg/m^3. Studies conducted to ascertain the lifetime of aerosols in air suggest that particles less than 5 μm in diameter remain suspended longer than 100 hr (Esmen and Corn, 1971), although removal by rainfall may shorten retention time. The biological activity associated with anthropogenic pollutants, and the exposure to higher concentrations due to localized emissions, render them a pollution problem of major concern. This study outlines chemical and biological testing procedures for both POM and VPO compounds in ambient air.

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Sample Collection

To provide the required amount of particles for combined bioassay and chemical analyses, the use of a Massive Air Volume Sample (MAVS) was necessary. It was originally anticipated that a 10 day sampling period would supply about 5 g of respirable particulate with an average extraction efficiency of 10% organics per unit weight of particulate sample. This 5 g sample would therefore provide approximately 500 mg of extractable organics, a sufficient amount for bioassay and chemical characterization. Unfortunately, the MAVS sampler was less than satisfactory in its performance. For those field trips in which the MAVS was utilized by RTI personnel, satisfactory particulate sample amounts were obtained in only two cases (Lake Charles, LA and Houston, Texas). For the Houston, Beaumont, Upland, and Elizabeth sampling trips, the ionizer failed to operate, and collection of the smallest size fraction (<1.7 μm) was achieved only by impaction. When the ionizer was operative, only minimal charge (0-7 mA) for the electrostatic plates was obtained. Problems were also encountered with the high voltage power supply, the electrostatic plate holder, the flow director plates and the sampler transformer. The net result was the collection of much less particulate sample than was expected, which forced the implementation of sampling regimens with variable and unknown collection efficiencies. These factors imposed severe constraints on bioassay mutagenicity testing in the Ames/*Salmonella* assay, which prevented the acquisition of more meaningful, quantitative mutagenicity results. In addition, the sizing capability of the MAVS was not always efficient. Normally, the smallest size fraction (<1.7 μm) contained the largest amount of extractable organic compounds per unit mass of particulate, but in some cases the extractable organics were greatest for larger size fractions. Scanning electron micrographs of such size fractions showed a much greater variation in particulate size among a given size class than would be expected, but this may have been caused by aggregation after sizing.

These problems have since been corrected, and the MAVS has been effectively utilized by the EPA to collect sufficient sample sizes (>50 g of particles) in New York City, Los Angeles, and Philadelphia (Lewtas et al., 1982; Lewtas and Claxton, 1981). This large amount of particulate sample is necessary for adequate initial mutagenicity testing with the *Salmonella* assay, and for chemical identification of signature mutagenic and carcinogenic compounds. The MAVS offers an additional advantage over other available samplers in the collection of ambient air particles. The ability of the collection device to size-fractionate particles is important and necessary in the toxicological evaluation of air particles because the smaller sized particles (<1.7 μm) are: (1) more easily inhaled; (2) have a longer residency time in ambient air; (3) are deposited more deeply in the lung and are therefore more difficult to remove; and (4) the smaller size particles present more surface area per unit weight for attachment of organic compounds than do larger sized particles. In addition, the MAVS can collect a gram of particulate sample in a 24 hr sampling period when operating properly. This ability of the sampler to collect gram quantities of sample, and to size-fractionate air particles, makes the MAVS the collection device of choice in ambient air particle sampling and testing program (Foord et al., 1975; Lippman et al., 1979; Natusch et al., 1974; NAS, 1974; NRC, 1979; and Schlesinger and Lippman, 1978).

Chemical Fractionation

The complexity of ambient air particulate extracts dictated a need for sample simplification prior to biological screening or chemical characterization. Chemical fractionation of the crude solvent extract into general chemical-classes before mutagenicity testing offered the following advantages over testing a crude extract: (1) elimination of toxic and/or antagonistic effects in the crude sample that may mask mutagenic effects (Epler et al., 1979; Pelroy and Peterson, 1979); (2) isolation of specific mutagenic chemical-class(es) within the crude extract (Pellizzari et al., 1979); (3) the ability to chemically identify the signature mutagenic compounds in the active chemical-classes (Hughes et al., 1980); and (4) the ability to investigate synergistic/comutagenic and antagonistic effects within and between these different chemical-classes (Ashby and Styles, 1978; Slega et al., 1978).

The most common techniques for a single step fractionation procedure involve either column chromatography (Hill et al., 1977) or solvent partition (Towika et al., 1977). The former method separates material on the basis of compound polarity while the latter procedure utilizes compound solubility. Open column procedures by surface active sorbents (silica, alumina, etc.) are slow, difficult to reproduce, and permit sample decomposition (Hughes et al., 1980). In contrast, solvent partition is mild, relatively rapid, and can be utilized to separate compounds into distinct classes. The scheme of Lee et al. (1976) was therefore adopted and modified for chemical separation of crude solvent extract from ambient air particulates into general chemical-classes. The fractionation procedure is described in Section 4 (Figure 2).

The solvent partition sequence served to simplify an otherwise extremely complex mixture. Each of the five fractions from the scheme represented a collection of compounds of like chemical (acid and base fractions) or physical (nonpolar and polar neutral, and PNA fractions) properties. These fractions were substantially enriched in certain types of compounds (depending on partition coefficients), but were not entirely composed of a single class of materials. Thus a typical PNA fraction consisted largely of PNAs, but also contained other materials which resulted from spillover from other fractions. For example, PNA compounds such as benzo(a)pyrene were identified in the PN fraction from Baton Rouge, LA.

Spillover occurred for a variety of reasons. For a given fraction to contain one type of compound would mean that the partition coefficients between compounds were different to the extent of the detection limits for analysis, and that the partition process was unaffected by the compounds present. These conditions were not realized in practice due to compounds which possessed mixed functional groups, and to complex interactions between the solutes and between a solute and the solvents.

The purpose for which a partition process was employed (*i.e.*, a simplification of a complex mixture prior to bioassay or analytical analyses) was realized. Results from validation tests and field samples confirmed the overall efficacy of the procedure (see Sections 4 and 5).

Further investigation should be performed on the chemical fractionation scheme developed under this program in regard to the spillover of compounds into the basic fraction, and spillover of PNA compounds into the PN fraction. In addition, reconstitution experiments with a fractioned field sample should be performed to determine the effect of chemical fractionation on the mutagenic potential of the crude extract. A reconstitution experiment would also permit investigation into synergistic/antagonistic compounds in complex environmental mixtures.

Other analytical techniques, such as open column chromotagraphy, preparative HPLC and TLC, were not examined due mainly to the lack of adequate sample size. These techniques should also be investigated, especially the HPLC technique, which may resolve the PNA/PN spillover problem.

Sonication was chosen as the procedure for removal of adsorbed organics from particulate samples primarily because of the mildness and speed of the procedure. It has been shown (Subcommittee 5, 1975) that sonication with cyclohexane was more efficient for the removal of PNAs than was Soxhlet extraction with benzene. Soxhlet extraction was thus not employed for any particulate samples collected under this program. One sample of particulate was analyzed for total organic carbon (TOC) after cyclohexane/methanol sonication, and was found to contain less than 10% TOC. Sonication was thus effective for removing approximately 90% of the total organic material from the inorganic particle matrix. No other solvent or solvent mixture was tested for efficacy of removal of organics from particulate samples under this program, but should be investigated further.

Mutagenicity of Ambient Air Particles

Little information existed on the potential mutagenicity of ambient air when this program was initiated in 1977. Earlier studies on organic materials extracted from air particles in industrialized areas demonstrated that they contained suspected mutagenic and carcinogenic compounds, which were identified by chemical methods only (Hoffman and Wynder, 1977; Fishbein, 1976). However, with the development of the Ames/*Salmonella* mutagenicity assay (Ames et al., 1975b), it became possible to evaluate the mutagenic potential of ambient air particles in a cost- and time-efficient manner. The coupling of this assay system with chemical identification of the signature mutagenic compounds on air particles offered a powerful tool for toxic evaluation of ambient air.

Consequently, this program utilized a multidisciplinary chemical-biological approach to develop a testing protocol for identification of mutagenic compounds on ambient air particulate samples. The first sample tested under this program was from South Charleston, West Virginia

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(Tables 11 to 13). This first sample tested for mutagenicity utilized a thirteen class chemical fractionation scheme (Figure 3) and the qualitative spot test (Ames et al., 1975b), with the five standard *Salmonella* tester strains (TA98, TA100, TA1535, TA1537, and TA1538), both with and without a S9 metabolic activation system. Mutagenic activity was detected in the organic bases, acids, and aromatics. Each of the five tester strains gave a positive response with at least one of the active fractions and only the aromatics required metabolic activation. In this West Virginia sample, GC/MS/Computer analysis identified 2-nitro- 4,5-dichlorophenol, a direct-acting mutagen in the polar bases (Wang et al., 1978). Fluoranthene, pyrene, benz(a)anthracene, and benzo(a)pyrene were identified in the aromatic fractions. All of these compounds have been identified either as co-carcinogens (Van Duuren, 1976) and/or mutagens requiring metabolic activation (McCann et al., 1975). These preliminary results indicated that the Ames assay could detect mutagenic activity in small sample amounts of ambient air (as a complex mixture), both for direct-acting mutagens and promutagens (requiring S9). In addition, it was demonstrated that the fractionation and analysis scheme was capable of identifying broad chemical mutagenic classes within these mixtures. It was concluded that chemical fractionation of the crude complex sample was both useful and necessary to accomplish adequate biotesting and chemical identification.

Analysis of the West Virginia sample delineated the problems of inadequate sample size and insufficient chemical resolution with the 13 class-chemical fractionation scheme. Consequently, a simpler fractionation scheme was developed (Figure 2), which increased available sample size in the chemical-class fractions and reduced spillover from one fraction to another. The minimum sample amount required for initial analysis of ambient air particulate was established as 30 g. This amount agrees with conclusions of other air pollution studies (Pitts et al., 1977; Talcott and Wei, 1977). The six fraction scheme was verified for biological and chemical efficiency (Section 4). It was further demonstrated that each chemical-class fraction can contain hundreds of compounds (Pellizzari et al., 1979), with only a small number of these compounds responsible for mutagenic activity (Table 45).

An additional technique was developed to optimize mutagenicity information in the Ames/*Salmonella* assay with small sample size (Section 4 and Appendix C). This assay, the agar well diffusion assay, required only 10 mg for initial testing, whereas the standard plate incorporation and spot techniques required 50 and 20 mg respectively (Ames et al., 1975b). The development of a refined technique for small sample size was necessary because even this small amount (10 mg) was available in only four of the ten fractions from Baton Rouge (Table 20). The agar well diffusion assay is similar in principle to the immunodiffusion technique of Ouchterlony (1968). The parameters of this assay were defined and the technique was verified with known mutagens (Appendix C). The utility of the agar well test for evaluation of mutagenic potential was demonstrated in the second West Virginia sample (Tables 15-17), where the well, spot, and plate incorporation assays were compared. These data suggested that results from the spot and well test were similar and that PNA type compounds (such as benzo(a)pyrene, or similar hydrophobic compounds) would not be detected with the spot or agar well tests.

Based on the above considerations, a bioassay priority testing scheme was developed for air particulate samples (Table 4). The scheme was based on total sample size. The assay-of-choice for initial mutagenic evaluation was the plate incorporation assay when sample size was above 10 mg. When sample size was below 10 mg, the agar well test was utilized on all chemical- classes except the PNAs, for which a plate incorporation test with S9 and TA98 was utilized. A tester strain priority scheme was the developed for both assay techniques. The agar well test increased the mutagenicity information obtained under this program. When coupled with chemical fractionation and identification, the agar well assay facilitated rapid evaluation of the mutagenic potential of ambient air, and helped define a minimal testing protocol. The plate incorporation assay, with a reproducible dose response mutagenicity curve, remained the assay-of-choice when sample amounts were sufficient and when weak mutagens were present.

The analysis of Baton Rouge particulate samples (Section 5) utilized the agar well assay, the bioassay priority testing scheme and the six-fraction chemical fractionation scheme. Both direct-acting mutagens and promutagens were identified in this sample for the <1.7 μm particles, and both frameshift and base substitution mutagens were identified. A dose response mutagenicity study was conducted on the PN fraction. A linear dose response relationship was demonstrated from 150-1,000 μg/plate; activity required S9 addition with strain TA98 (Figure 18). Chemical identification of signature mutagens in each fraction (Table 45) correlated with the mutagenic activity detected (Table 20 and 21). Mutagenic activity was detected in the PNA, PN, acid and base fractions, and known mutagens were identified in each class. The NPN fraction was nonmutagenic; no mutagenic compounds were identified in this class. It should be noted that the NPN fraction did contain over a hundred compounds, which were mostly straight chain hydrocarbons (*e.g.*, hexane). None of these compounds were mutagenic, which correlated with the generally negative mutagenic potential of the NPN class. The identified mutagens in the PN class were attributed to PNA type compounds, which resulted from the inability of the chemical fractionation scheme to *absolutely* separate compounds with similar chemical properties. An alternate chemical technique, such as HPLC, may resolve this separation/spillover problem. This data suggested that the most sensitive assay technique to detect mutagenic potential was the pour plate incorporation assay. However, the agar well test was useful and necessary when sample size of the chemical-class fractions was small.

The utility of the agar well test to evaluate mutagenic potential was again demonstrated with the first Lake Charles, Louisiana sample (Table 23). Limited sample amount precluded dose response testing with the plate incorporation assay. However, the qualitative agar well test identified the mutagenic PN fraction from this first sample (Table 23), which was confirmed in a second, more quantitative sample from Lake

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Charles (Table 24). In addition, the <1.7 μm sized fraction from the first Lake Charles sample (Table 23) was qualitatively more mutagenic, in more chemical-class fractions, and with more *Salmonella* tester strains than was the >1.7 μm sample as determined by the agar well assay.

The increased mutagenic potential of the smaller sized ambient air particles (*i.e.*, <1.7 μm) was also demonstrated in the Upland, California sample (Table 34). Three size classified fractions were collected (<1.7 μm, 1.7-3.5 μm, and >3.5 μm) at the Upland site. The acid, PNA, and PN fractions were mutagenically active, with the qualitative mutagenic activity decreasing as the particle size increased. Increased mutagenic activity has been demonstrated on smaller sized particles of fly ash (Crisp et al., 1978) and air particle samples from Chicago (Commoner et al., 1978) and Durham, NC (Talcott and Harger, 1980). These results confirmed that ambient air collection devices should have the ability to discriminate particle size.

The second Lake Charles sample (Table 24) demonstrated that a major advantage of the chemical fractionation scheme was the concentration of mutagenic components from the complex mixture of organics in the solvent extract from air particles. The crude extract at 1,000 μg/plate produced 127 colonies/plate; the PNA fraction at 500 μg/plate produced 1007 colonies/plate. The fractionation scheme therefore allowed detection of a highly mutagenic compound from a number of compounds, which would not have been detected had only the crude extract been tested. The PNA fraction, although a minor component (1.8%) of the total extract, was the major mutagenic component for TA98. In addition, the chemical fractionation scheme allowed meaningful chemical characterization to be accomplished. Chemical analysis of the crude solvent extract was not possible before fractionation. However, after fractionation, known mutagens and carcinogens were qualitatively identified in their respective chemical-class (Table 45). The PNA fraction, which was mutagenic for both Lake Charles samples, also contained the greatest number of known mutagens.

Another advantage of the chemical fractionation scheme was the ability to obtain quantitative information on the varying amounts of chemical-class fractions from each site. The total amount of atmospheric pollution can change with atmospheric conditions and time of year (Commoner et al., 1979), and this can change the mutagenic potential. Comparisons of the fractions from these two Lake Charles samples indicated large percentage fluctations in various chemical fractions from the two samples tested (Table 22). These fluctations in percentage of chemical classes may indicate varying industrial output. The amount of each chemical-class within the crude extract must also be known to calculate the specific mutagenic activity in revertants/μg of particulate sample.

The results from the Houston, Texas particulate sample (Table 29) demonstrated the ability of the chemical fractionation scheme to unmask mutagenic activity (in the acid and PNA fractions) that was not demonstrated when the crude organic extract was tested. The unmasking of mutagenic potential in this analysis was probably due to a concentration

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of relatively minor components (by weight), since the mutagenic acid fraction accounted for less than 1.0% of the crude extract and the mutagenic PNA fraction accounted for less than 3% of the crude extract (Table 28). Likewise, the unmasking of mutagenic activity was also evident in the second Houston sample, where the total extractable organics demonstrated no mutagenicity with TA98, but activity was demonstrated in all five chemical-classes (bases, acids, PNA, PN, and NPN) after fractionation (Table 31).

The analysis of the second Houston sample highlighted some important steps when testing complex environmental mixtures (Table 31). The PNA fraction should be tested both with and without S9 addition, since activity was demonstrated for the PNA fraction without S9, and mutagenic activity was inhibited in the PN fraction with TA1535 when S9 was added (Table 31). These results strongly suggested the presence of both direct-acting mutagens and promutagens. Therefore, all samples and fractions should be tested both with and without S9 addition. The amount of S9 per plate at any given dose was also demonstrated to be important with the second Houston sample, since mutagenic activity increased 3-fold in the PN fraction with TA1535 when the S9 amount was increased from 3.0-6.0 mg of S9 protein/plate (Table 31). This result suggested that the S9 concentrations are important when testing unknown complex mixtures. Varying the amount of sample was also demonstrated to be an important factor. The PNA fraction was not active at 100 μg/plate, but significant mutagenic activity was demonstrated at 500 μg/plate (both without and with S9 addition) and TA98 (Table 31). Consequently, double dose response testing (i.e., varying both the amount of sample and S9) should be performed on ambient air particulate samples.

The Elizabeth, New Jersey study (Table 37) produced results comparable to those in the six other geographical areas. The results shown in Table 37 demonstrated: (1) the mutagenic activity was primarily present in the PNA, PN, acid and base fractions; (2) the mutagenicity decreased as the surface area/unit mass of particulate decreased, i.e., as the diameter of the particle increased; (3) the fractionation scheme unmasked mutagenicity which was not seen in the total extractable organics (crude extract); (4) toxic effects present in the crude extract were reduced after fractionation; (5) PNA and PN fractions required metabolic activation while acids and bases (the other two active fractions) did not; the NPN fraction was not mutagenic (also see Beaumont Table 26); (6) both frameshift and base substitution mutagens were detected; and (7) sample size for the acid, base, and PNA fractions was less than 10 mg, but the agar well test allowed initial analysis of the total sample and its fractions.

Since 1977, the results discussed above have been demonstrated in other air pollution studies. For example, Pitts et al. (1977) collected air particulate at rural and urban sites in California and extracted the organics with sonication in equal parts of methanol, benzene, and dichloromethane. The particulate collected from the rural site was not mutagenic, but material from all eight urban locations was mutagenic without S9 addition, with a linear dose response for Salmonella strains TA98, TA1538, and TA1537. Thus, the mutagenic components present were probably not of the BAP class, which require S9 activation. In another case, mutagenic activity was demonstrated in a California air sample and S9 activation was required (Flessel, 1977).

In Japan, ambient air was sampled at six different residential and industrial sites (Tokiwa et al., 1977). The organic fraction from ambient air particles (extracted with methanol) collected in Ohmuta (an industrial city) was mutagenic and exhibited a linear dose response from 100-400 μg with *Salmonella* strain TA98. A sample collected from a residential area (Fukuoka City), required from 370-1970 μg/plate to generate a positive mutagenic response. The mutagenicity was approximately three times greater for the sample collected from the industrial city, and a linear dose response was observed for both samples. These researchers identified the compounds in the methanol extract with an alumina column and GC/MS analysis. A majority of the compounds identified were polycyclic aromatic hydrocarbons (PNAs), such as benzopyrenes, anthracenes, fluorenes, and dibenzo isomers of these compounds. These studies in Japan demonstrated that air particulate samples near industrial sites possessed higher mutagenic potential than samples from rural sites. Both S9 activated (BAP) and direct-acting (non-BAP) mutagenic components were present, which agreed with the California studies of Pitts et al. (1977) and Flessel (1977) described above.

Talcott and Wei (1977) collected air particulate on glass fiber filter and extracted the filter with acetone in a Soxhlet apparatus. The extracted material exhibited the highest activity with TA100 and required S9 metabolic activation. Direct-acting mutagens were also detected with TA98 and TA1537. Activity was again attributed to at least two types of mutagens: a PNA and a non-PNA fraction. The view that PNA compounds are not the sole (or major) mutagenic factor associated with air particulate was further supported by the studies of Dehnen et al. (1977). Here mutagenic activity was predominantly produced by compounds other than PNAs, since the observed mutagenicity did not require S9. Dehnen used a chemical fractionation scheme to divide his crude air particulate sample into cyclohexane- and methanol-extractable fractions. An alumina column was employed to further fractionate the cyclohexane extract into a purified cyclohexane fraction (containing PNA-type compounds) and a 2-propanol fraction (containing azo-heterocyclic compounds). The highest mutagenic response was found in the 2-propanol and methanol extracts; neither fraction contained PNA-like compounds.

Tokiwa et al. (1977) combined a chemical fractionation scheme with an Ames detection system for air pollution studies. After solvent extraction, fractionation of the sample into chemical-classes permitted isolation of the mutagenically active chemical-classes within the crude mixture, and allowed chemical identification of the mutagenically active compounds within the less complex chemical-classes. In some cases, fractionation appeared to reduce or remove toxic effects, which otherwise would have prevented expression of mutagenicity. The synergism or antagonism of the individual components in the crude mixture can thus be investigated. Pelroy et al. (1979), observed such effects with shale oil and its fractions, as did Epler et al. (1979), with fossil fuels and their fractions.

The results of air pollution studies by Teraniski et al. (1978) agree with the above results. Air particulate was collected, Soxhlet extracted in benzene, and fractionated into five fractions by a chemical partition scheme: acids, bases, aliphatics, polynuclear aromatic hydrocarbons, (PNA), and oxygenated (PN) fractions. The Salmonella assay (TA98) detected activity in the benzene-soluble organic crude fraction and in the acidic, polyaromatic and oxygenated fractions. The only fraction that did not require S9 liver microsomal activation was the acidic fraction. A minimum of 250 μg of sample was required to produce a positive result, with a linear dose response effect from 250 and 1000 μg/plate. Activity in the crude fraction was detected on TA1537, TA1538, TA100, and TA98, which indicated the presence of both frameshift and base substitution mutagens. Mutagenicity increased 2-fold with the addition of S9. The aromatic and oxygenated (PN) fractions had an absolute requirement for S9 metabolic activation, whereas the mutagenicity of the acidic fraction did not vary significantly with S9 with TA98. The amount of base collected was small, but the mutagenicity per unit mass for this fraction was higher than any other fraction.

Talcott and Harger (1980) observed that most of the mass and mutagenic activity of an air particulate sample collected in Durham, N. C. was in fractions less than 2 μm in diameter. An Andersen 2000 sampler and the Ames/Salmonella assay were utilized. Ames testing of acetone extracts of sized particulate samples (<2 μm, 2.1-3.3 μm, 3.3-4.7 μm, 4.7-7 μm, and 7-11 μm) revealed that 72% of the mutagenic activity resided in the <2 μm-size particulate. The agents responsible for this activity on TA98 did not require metabolic activation. The investigators observed that the mutagenic activity correlated with the presence of electrophiles, detected by nitrobenzylpyridine or by DC polarographic analysis, and concluded that the mutagenic activity was due to direct-acting alkylating agents. Commoner et al. (1978) employed the Salmonella assay to analyze 15 Chicago air particulate samples collected from a heavily industrialized area of steel mills and coke-ovens, and found mutagenic activity in the presence and absence of S9. Salmonella strain TA1538 was the most sensitive strain to these mutagens, and benzo(a)pyrene and benzo(e)pyrene were identified in the samples by chromatography and mass spectrometry. A benzene/hexane/isopropanol solvent-extract was utilized. Results suggest that Commoner may be selecting polar neutral compounds with the benzene/isopropanol/hexane solvent system and PNAs with benzene. The water-soluble fraction was negative. The requirement for S9 exhibited seasonal variation; mutagenic activity was present in April and August without S9, but metabolic activation was required in December.

The mutagenicity results from the above studies have led to the development of more quantitative air pollution studies. Recently, Tokiwa and co-workers (1980) utilized their earlier results (Tokiwa et al., 1977) to perform a quantitative investigation of the mutagenicity of air particles in Japan. Solvent fractionation of gram quantities (1-6) of sample from an industrial and rural city was performed and fractions were tested for mutagenicity with Salmonella strains TA98 and TA100. Samples were size-fractionated with an Anderson Sampler. Mutagenic activity was reported in revertants/plate/m^3. Significant activity was demonstrated in the basic, acid, and PNA fractions. The amount of BAP correlated with the mutagenic activity of the industrial site but did not correlate with the mutagenic activity of the rural site. Linear dose response curves for mutagenicity were demonstrated from 0-200 μg/plate for the industrial site, with mutagenic activities approximately twice as great for the industrial site as for the rural site (33 rev/plate/m^3 vs. 15 rev/plate/m^3). TA98 was the preferred tester strain. Extraction efficiency was high (25%), but reconstruction experiments after fractionation accounted for only 58% of the mutagenic activity detected in the crude extract. Potent polycyclic mutagens were identified in the PNA fraction. The smallest sized particles (0.3-1.0 μm) possessed the most organic extractable material, and demonstrated the greatest mutagenic potential of any size fraction (44.8 rev/m^3). The >9.5 μm particles were the next most mutagenically active size fraction (8.9 rev/m^3). This study also emphasized the fact that total mutagenic potential of ambient air particles could not be attributed solely to the PNA fraction; the acid and basic fractions were also significantly mutagenic.

In summary, the quantitative Tokiwa study (Towika et al., 1980) described above agreed with other investigations and with the results generated within this report. Although the results generated under this program were qualitative, they did agree with the quantitative studies described above. This EPA developmental program evaluated and tested a Massive Volume Air Sampler (MAVS) and demonstrated the necessity for size-fractionation of air particulate before bioassay. The efficiency of chemical fractionation methods to separate crude extracts into simpler chemical-class was evaluated and was shown to facilitate both chemical and biological analysis. A modified plate assay (agar well diffusion technique) for the Ames/Salmonella assay was developed and tested, and was found to greatly aid in the evaluation of mutagenicity of air particulate when sample size was limited. A bioassay priority testing scheme was developed and utilized to obtain the maximum amount of information from each sample. Probable mutagenic classes and chemical compounds were qualitatively identified from seven geographical sites.

Chemical Identification of Signature Mutagens and Carcinogens in POM

The use of techniques other than capillary GC/MS was not actively pursued because alternative chemical procedures such as HPLC or open column chromatography do not possess adequate resolution capability (Novotny et al., 1976; Hughes et al., 1980).

Capillary GC/MS was the technique utilized for all chemical characterization since other techniques, such as HPLC, did not possess the chromatographic efficiency of capillary GC. The limited amount of sample (after removal of suitable aliquots for bioassay) placed severe restrictions on the utilization of any combination of procedures. The specificity of GC/MS (Bartle et al., 1976; Alford, 1977; Cautreels and Van Cauwenberghe, 1977; Daisey and Leyko, 1979) renders this approach most effective for samples that contain volatile species. For nonvolatile or thermally labile compounds (*e.g.*, polar neutrals), other

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techniques are indicated; HPLC is an obvious choice. The specificity of a newly developed technique, Fourier transform infrared spectrophotometry or FTIR, should also be investigated for vapor-phase organics (Hanst, 1978).

Signature mutagens and/or carcinogens were identified in most mutagenically active chemical-classes. It should be noted that the NPN class, which was generally nonmutagenic, did not contain many identifiable mutagenic compounds. In addition, although the mutagenic potential of PNA type compounds is well documented (Brooks, 1977; Teranishi et al., 1978), mutagenic activity and mutagenic compounds were also identified in the PN, acid, and base fractions (Table 45).

Compounds in the chemical-class fractions were identfied by a GC/MS/Computer system; detected mutagens are listed in a summary table (Table 46). Some of the compounds identified in the fractions were co-carcinogens, such as fluoranthene and pyrene (Van Duuren, 1976). Since each chemical-class contained from 30-150 compounds, many of which could only be identified by empirical formulae (Little et al., 1978), the crude organic extract probably contained more than 300 compounds (Pellizzari et al., 1979). Many PNA-like compounds were detected in the PN fraction; it is possible that the apparent mutagenicity of this fraction was due to spillover from the PNA fraction.

The overall mutagenic potential correlated with the number of identifiable mutagens. However, this correlation was performed on a qualitative basis only, and does not account for comutagenic and synergistic effects (Slega et al., 1978; Tokiwa et al., 1980). Additional research to quantitatively correlate mutagenic potential with chemical mutagens in complex ambient air mixtures is needed.

VAPOR-PHASE ORGANICS (VPOs)

Techniques Developed to Evaluate the Mutagenicity of Ambient Air VPOs

The main thrust of the VPOs study was to develop a biological/chemical interface procedure that would allow for meaningful mutagenicity testing of vapor-phase organic materials. The collection procedure with Tenax®, already developed and utilized routinely in our laboratory, was adopted and utilized for interface development (Pellizzari, 1975, 1979). Tenax® cartridges, for collection of VPO field samples and subsequent chemical identification of vapor-phase mutagens, were the method-of-choice over other methods of collection (*i.e.*, charcoal) for the following reasons: (a) superior ability to discriminate against water uptake and preferentially collect and concentrate the vapor-phase organics of interest; (b) low background contribution from Tenax® in subsequent analysis; (c) minimal decomposition or polymerization of the sample during collection and recovery; (d) quantitative collection efficiency and recovery efficiency of trapped vapors from Tenax®; (e) high breakthrough volumes (*i.e.*, high uptake ability of VPO mutagens); and (f) no *in situ* formation of artifacts with Tenax® (Hughes et al., 1980).

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TABLE 46 . COMPOUNDS IDENTIFIED BY GC/MS IN AIR PARTICULATE FROM 7 SITES THAT ARE EITHER MUTAGENS (McCann et al., 1975) AND/OR CARCINOGENS (Sawicki, 1979)

PNA	Anthracenes, benzo(a)pyrenes, benz(a)anthracenes, fluorene, pyrene, perylenes, phenylnaphthylamine, napthalene
PN	Benzo(a)pyrene, saturated hydrocarbons, fluoranthene, pyrene, chrysenes
Acids	Phenol, cresol, benzenes
Bases	Quinolines, nicotine, acridines, 2-nitro-4,6-dichlorophenol
NPN	No compounds identified

Plural terms indicate isomers such as benzo, dibenzomethyl, and fluoro isomers of the parent compound.

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Quality control checks of the overall approach for collection of VPOs employed various standards, and results were acceptable (Figures 7 and 8). However, validation experiments for certain aspects of the methodology are required before field samples can be processed. The overall recovery of a split field sample, as determined by comparison with direct thermal desorption/GC/MS, should be ascertained in replicate. The storage requirements for the VPOs in ethylene glycol/water/ethanol needs further definition. Preliminary studies suggested significant losses (~40%) of ethylene oxide when stored at -80°C for one week, and minor losses of propylene oxide (~8%) and butylene oxide (~0%) when stored under the same conditions.

As discussed earlier (Section 4), no mutagenicity field testing was performed due to the lack of an adequate quantitative technique to measure vapor-phase organic mutagens in the Ames/Salmonella assay. The standard pour plate method is not adequate to detect mutagenicity of compounds with high vapor pressure because of sample loss due to placing the sample in agar at 45°C and subsequent incubation at 37°C. Substances which boil below the temperatures will volatilize before the system has an opportunity to form mutagenic compounds. Previous studies outlined the difficulties inherent with research on the mutagenic potential of organic vapors. For example, Loprieno et al. (1976) reported a positive mutagenic effect with yeast and vinyl chloride at relatively high concentrations (5 and 50%) in liquid suspension. However, the S9 was stable for a maximum of only 60 min in liquid culture and may have been a limiting factor for full mutagenic expression. Malaveille et al. (1975, 1977) detected mutagenicity of vinyl chloride to Salmonella strains TA1530, TA1535, G46, and TA100 at concentrations of 2 and 20% v/v in a dessicator. Mouse liver, induced with phenobarbitone and stabilized with EDTA, was used in these studies. Quantitation of this method was difficult because of the high volatility of vinyl chloride.

Rannug et al. (1976) utilized liquid preincubation of the bacteria and S9 (1 hr at 25°C) to study the metabolites of vinyl chloride and demonstrated that chloroethylene oxide and chloroacetaldehyde were the major metabolites. Quantitation was difficult in this study because of the low aqueous solubility and high volatility of vinyl chloride. Rosenkranz et al. (1979) utilized a modified spot test to detect vapors; discs impregnated with volatile sample were incubated at 4°C for 2 hr, removed, and incubated at 37°C for 48 hr or the discs were immediately placed in sealed plastic bags and incubated at 37°C. A limitation of this method was that the non-diffusible (normally aqueous-insoluble) test agents can not be detected.

The above results suggested that biological analysis of complex vapor-phase organic field samples in the laboratory would be considerably more difficult than analysis of single vapors. This was due to the low amount of sample obtainable, degradation of sample, and lack of adequate techniques for mutagen detection.

The approach employed to evaluate the mutagenic potential of ambient air vapor-phase organic was multidisciplinary. The collection techniques for vapor-phase materials presented an additional technical problem, but a validated method with Tenax® cartridges for vapor collection and chemical analysis developed by Pellizzari (1979) was employed. A literature survey was conducted to investigate the relationship between apparent mutagenicity and vapor pressure of mutagens (Table 47). For example, the melting point and mutagenic potential of 2-aminofluorene was high, yet the mutagenic potential and boiling point of vinylidene chloride was low. The apparent low mutagenic potential of low boiling point compounds such as propylene oxide and vinylidene chloride may therefore be due to improper technique, volatility or instability.

Consequently, the development of a quantitative technique to measure the mutagenic potential of vapor-phase organic compounds in the Ames/Salmonella assay was investigated. Initial studies with both the hollow-fiber filter and charcoal adsorption techniques had demonstrated that these methods were inappropriate due to the inability of hydrophobic compounds to diffuse from the fibers into the agar (Section 4). These studies led to the investigation of the liquid suspension assay of Yahagi et al. (1975). In liquid suspension, diffusion would not be a technical difficulty; incubation time, and temperature could be varied to optimize the association between bacteria and compound; the volatile gases could not escape from a closed system. In addition, the amount of test compound in the gaseous and liquid state could be varied by adjusting the amount of air space and the temperature since Milvy and Garro (1976) had shown that styrene oxide was more effectively detected in the vapor state rather than in the liquid state. Liquid suspension bioassays also would be compatible with thermally desorbed/cryogenically frozen, Tenax® trapped vapor- phase organic field samples.

The preincubation protocol developed under this program (Table 7) was modified from the method originally developed by Yahagi et al. (1975) to optimize sensitivity. Log-phase cells were utilized in place of stationary-phase cells since Guttenplan (1979) had demonstrated that log-phase cells were 3 times more sensitive to DEN than were stationary-phase cells. The increased sensitivity of the log-phase cells was due to their inability to repair premutagenic DNA lesions. Agar was added to the preincubation vial to stabilize the S9 since Malaveille et al. (1977) have demonstrated that agar has a physical effect on S9 activity. Trace amounts of additional histidine and biotin were added to keep the cells metabolically active (Ames et al., 1975). Optimal preincubation time was determined to be 30 min; Yahagi et al. had demonstrated that the mutagenic activity of DNM and DEN increased up to 50 min of preincubation. Preincubation temperature was empirically determined at 37°C, since this temperature would volatilize the vapor-phase compounds in a closed system and would keep the bacteria metabolically active. Experiments to investigate the above technical parameters were discussed in Section 4.

The preincubation technique was developed and compared to the standard pour plate technique. Results were obtained with six active vapor-phase mutagens: butylene oxide, propylene oxide, ethylene oxide, ethylene dibromide, styrene oxide, and vinylidene chloride. The standard

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TABLE 47. RELATION BETWEEN VAPOR PRESSURE OF SELECTED CARCINOGENS AND APPARENT MUTAGENICITY AS DETERMINED BY AMES/SALMONELLA ASSAY

	Mutagenicity (revertants/nmole)	Carcinogenicity	Melting Point (°C)	Boiling Point (°C)
Methyl bromide	[a]	[a]		4.6
Vinyl chloride	+weak	+		13.9
Propylene oxide	[a]	[a]		34/35
Vinylidene chloride	+weak	+		31.7
Chloroprene	weak			51.4
Diepoxy butane	0.12	+		60
Carbon tetrachloride (tetrachloromethane)	<0.001	+		76.74
Bischloromethylether (BCME)	+	+		104
Ethylene dibromide (EDB)	0.06	+		131
Dimethynitrosamine	0.02	+		152
Diethylnitrosamine	0.01	+		177
Benzyl chloride	0.02	+		179
Styrene oxide	0.37	Not Known		145
Safrole	<0.01	+	11	233
Cyclophosphamide	1.4	+	41-45	
Urethane (ethyl carbamate)	0.0006	+	49	180
Hydrazine sulfate	weak	+	85	
2-Aminofluorene	11300/10	+	131-132	
Dieldrin	<0.03	+	175	
2-Aminoanthracene	510	+	238/241	
Ethionine	0.02	+	decomp. 272	

[a] Data not available. Mutagenicity and carcinogenicity data taken from McCann (1975).

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pour plate assay of Ames et al. (1975b) was tested with two different growth phases of Salmonella (stationary- and log-phase cells) to determine which growth phase was more sensitive. The pour plate technique was also compared to the preincubation assay (with log-phase cells) to determine the optimal VPO detection technique. Tandem toxicity assays (with each technique) were performed at each dose to determine viability. This was important since log-phase cells had a lower number of cells/ml, and were more sensitive to the toxicity of the test compounds. Toxic potential was then utilized to calculate revertants/10^6 surviving cells, which allowed the three techniques to be compared to each other without bias. Guttenplan (1979) has demonstrated that log-phase cells are more sensitive to the toxic effect of DEN than stationary-phase cells and that adjustment for viability was necessary to avoid bias when mutagenic potentials were compared. Results were tabulated and graphed for each sample with the three techniques (Section 5).

Results were statistically analyzed by the method of Myers et al. (1981) to evaluate the most sensitive and precise assay technique for detection of vapor-phase mutagens. This statistical technique determines the mutagenic activity as the initial slope ($\underline{S}$) of the test compound from the linear portion of the dose response curve. The estimate of the initial slope of the dose response curve ($\underline{S}$) can be compared with the slope of other compounds and this information can be utilized to rank and categorize the samples. The method provides a package of models from which the data analyst can select a functional form appropriate to the data at hand. The models are biologically based, with parameters having clear interpretations in terms of linear or higher order mutagenicity, saturation, and toxicity.

The model selected for the vapor-phase data assumed that the mean number m(x) of revertants at dose x (micrograms) has the form:

$$m(x) = (B + Sx)\, e^{-Tx}$$

The exponential term exp(-Tx) is included to account for cell death due to toxic effects of the compound. The term (B + Sx) can be regarded as a linear approximation to (i) a 1-hit mutagenicity model, or (ii) a first-order kinetic model. B represents the average spontaneous background reversion rate (solvent control at dose 0) and S is a toxicity-adjusted initial mutagenicity slope, in units of revertants per microgram. Compound mutagenicity may be categorized in terms of the magnitude of S and a one-tailed test of the hypothesis $S = 0$ against the alternative that $S > 0$.

The statistical procedure used for parameter estimation and hypothesis testing is based on weighted least square, whereby each data point is weighted inversely proportional to the estimated variance for that dose. Estimates obtained in this way tend to be more precise than unweighed estimates. The weighed least square regression analysis uses the nonlinear regression procedure (NLIN) of the Statistical Analysis System. The output of the procedure includes final estimates of the parameters (in this case, B, S, T), standard errors and 95% confidence intervals for

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the parameters. Under the above model, a compound was judged significantly mutagenic at the 2.5% level in a one-tailed test if the 95% two-tailed confidence interval for S was completed above zero.

Statistical results, calculated by the method of Myers et al. (1981) for six vapor-phase compounds tested are listed in Table 48, and are reported as initial mutagenicity slope ($\underline{S}$), coefficients of variance for S, and confidence intervals for $\underline{S}$. Overall the mutagenicity slope $\underline{S}$ for the vapor-phase compounds was highest in the preincubation technique with log-phase cells. This increased mutational frequency indicated that the preincubation technique with log-phase cells was more specific in determining $\underline{S}$ than the other two techniques. Overall, the sensitivity of detection was increased by a factor of three or more in the preincubation assay. In addition, the coefficient of variance of $\underline{S}$ was lower with preincubation, which indicated that the precision in determining $\underline{S}$ was greatest in the preincubation assay.

For each of the three procedures, a confidence interval for $\underline{c}$ was determined by fitting the relation $s^2 = \bar{x} + c\bar{x}^2$ to the empirical pairs ($\bar{x}$, x^2) of means and variances computed for each dose level with each technique with each compound. This was accomplished by regressing $y = s^2 - \bar{x}$ on x^2 with a forced zero intercept by the Statistical Analysis System (SAS) General Linear Models (GLM) procedure.

The results of this analysis are in Figure 32. Results for $\underline{c}$ were .003 ± .0012 for the preincubation assay with log-phase cells, .009 ± .0028 for the pour plate assay with log-phase cells, and .0155 ± .006 for the pour plate assay stationary-phase cells. The confidence interval for the preincubation log-phase procedure was entirely below those for the other two procedures, which suggested that the preincubation log-phase procedure was the least variable and therefore most precise procedure at any given dose level.

The statistical analysis should be qualified on at least two counts. First, it is not known whether the quadratic variance vs. mean relation employed was adequate. Also, the independent variable $\bar{x}^2$ in the regression is intended to estimate μ^2, and does so with some error. Ordinary regression assumes the independent variable is known without error.

To investigate whether the statistical model of Myers et al. (1981) was reasonable, statistical results were compared to results calculated by the method of Stead et al. (1981). Results are shown in Table 49. Both statistical methods verified that the preincubation assay with log-phase cells was the most sensitive technique and results from the two statistical techniques were similar.

The initial toxicity adjusted mutagenicity slope ($\underline{S}$), in units of revertants per microgram, is the key parameter for interpretation of Ames test results (Myers et al., 1981). Sensitivity in detection is indicated by a higher numerical value of $\underline{S}$. Styrene oxide, when tested in the preincubation technique with log-phase cells, had the highest initial slope value of all compounds tested (1.5374 rev/μg). Mutagenic

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TABLE 48. COMPARISON OF INITIAL MUTAGENICITY SLOPES (REVERTANTS/μg)[a] FOR SIX VAPOR-PHASE COMPOUNDS WITH THREE DIFFERENT BIOASSAY PROCEDURES

Procedure	Compound	Initial Mutagenicity Slope (S) in Revertants/µg	Coefficient of Variance for S (Std. Error of S/Estimate of S)	Confidence Intervals for S Lower Limit	Confidence Intervals for S Upper Limit
Preincubation: Log-Phase Cells	Ethylene Dichloride	0.0000	0.0100	-0.0214	0.0214
	Ethylene Oxide	0.1453	0.4081	0.0181	0.2726
	Styrene Oxide	1.5374	0.0772	1.2880	1.7867
	Ethylene Dibromide	1.0103	0.1368	0.7060	1.3146
	Propylene Oxide	0.6502	0.0837	0.5371	0.7633
	Butylene Oxide	0.4846	0.1028	0.3797	0.5894
	Vinylidene Chloride	0.8800	0.2000	0.4600	1.3000
Pour: Log-Phase Cells	Ethylene Dichloride	0.1752	0.4172	0.0172	0.3332
	Ethylene Oxide	0.3675[a]	0.2054	0.2069	0.5290
	Styrene Oxide	0.7984	0.1086	0.6162	0.9805
	Ethylene Dibromide	0.3435	0.2757	0.1446	0.5429
	Propylene Oxide	0.3315	0.1210	0.2480	0.4151
	Butylene Oxide	0.0336	10.1666	-0.6841	0.7513
	Vinylidene Chloride	0.8600	0.1300	0.6300	1.0900
Pour: Stationary-Phase Cells	Ethylene Dichloride	0.0583	3.2590	-0.1757	0.2924
	Ethylene Oxide	0.0879	0.5484	-0.0147	0.1906
	Styrene Oxide	0.4543	0.1688	0.2932	0.6155
	Ethylene Dibromide	0.6632	0.2835	0.2666	1.0599
	Propylene Oxide	0.0936	0.3162	0.0319	0.1553
	Butylene Oxide	0.0972	0.4717	0.0009	0.1935
	Vinylidene Chloride	0.4100	0.2700	0.1800	0.6400

[a] 0.1780 if outlier at 125 colonies for spontaneous backmutation rate is removed.

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Figure 32. Confidence intervals for the variance parameter C for the three procedures

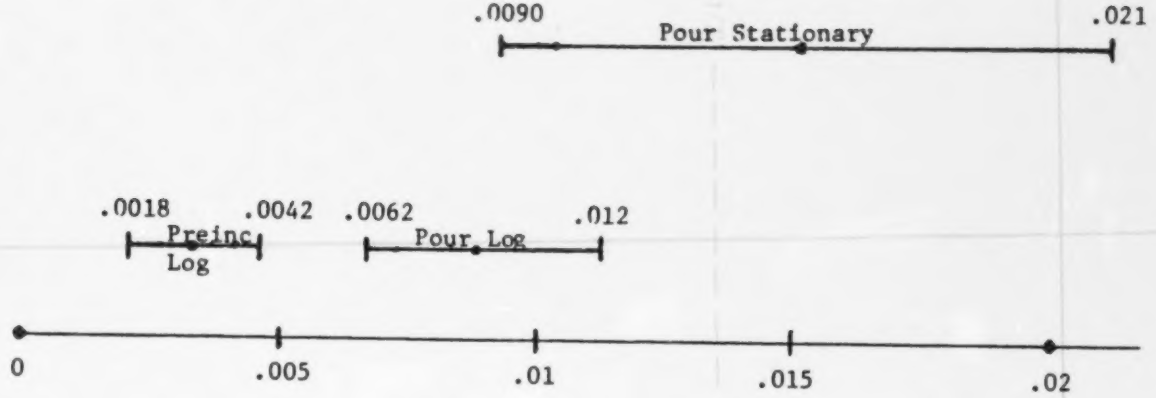

TABLE 49. COMPARISON OF INITIAL MUTAGENICITY SLOPES (REVERTANTS/µg) FOR SIX VAPOR-PHASE COMPOUNDS BY TWO STATISTICAL METHODS

Procedure	Compound	Initial Mutagenicity Slope (S) in Revertants/µg Myers et al. (1981)	 Stead et al. (1981)	Mutagenic Response from Model Myers et al. (1981)	 Stead et al. (1981)
Preincubation: Log-Phase Cells	Ethylene dichloride	0.0000	0.0000	-	-
	Ethylene oxide	0.1453	0.1464	+	+
	Styrene oxide	1.5374	1.5261	+	+
	Ethylene dibromide	1.0103	1.0498	+	+
	Propylene oxide	0.6502	0.6366	+	+
	Butylene oxide	0.4846	0.4768	+	+
Pour: Log-Phase Cells	Ethylene dichloride	0.1752	0.1750	+	+
	Ethylene oxide	0.3675	0.3677	+	+
	Styrene oxide	0.7984	0.8383	+	+
	Ethylene dibromide	0.3435	0.3665	+	+
	Propylene oxide	0.3315	0.3041	+	+
	Butylene oxide	0.0336	0.0383	-	-
Pour: Stationary-Phase Cells	Ethylene dichloride	0.0583	0.0582	-	-
	Ethylene oxide	0.0879	0.0902	-	-
	Styrene oxide	0.4543	0.4699	+	+
	Ethylene dibromide	0.6632	0.6810	+	+
	Propylene oxide	0.0936	0.0858	+	+
	Butylene oxide	0.0972	0.0982	+	-

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activity was considerably less in the pour-log (0.7984 rev/µg) and the pour-stationary assays (0.4543). Results suggested that the preincubation technique was generally more sensitive than pour plate technique and that log-phase cells were more sensitive than stationary-phase cells. Milvy and Garro (1976) tested styrene oxide by three methods: (1) placing a drop in the middle of the agar dish ("spot" test); (2) placing an aliquot in an agar overlay; and (3) placing an aliquot on a petri plate lid. Significantly more mutagenic activity was detected in the petri plate lid method, which suggested that styrene oxide was metabolized as a vapor and not as a liquid. The utility of the preincubation assay for detection of mutagens in the gaseous and liquid states was therefore demonstrated with styrene oxide. The amount of gaseous head space in the vial and the incubation temperature can be varied, which allows compounds to remain in both states simultaneously. Although styrene oxide was detected in the liquid phase under this preincubation protocol, significantly more activity may be detected by increasing the head space in the vial. Mixing of bacteria and compound could be facilitated by addition of sterile glass beads and by vigorous mixing. This aspect of the technique should be investigated further. Table 47 indicated that styrene oxide was mutagenic at 3.7 rev/µg; the lower activity seen here (1.54 rev/µg) may be due to a lower amount of the styrene oxide in the gaseous state. In addition, the ability to reproducibly detect this compound may be difficult due to its instability, since styrene oxide demonstrated activity of only 0.4543 rev/µg in the pour plate assay with stationary-phase cells (Table 48).

The mutagenic potential of two closely related compounds, ethylene dichloride (EDC) and ethylene dibromide (EDB), was investigated (Section 5). EDC was found to be nonmutagenic with an initial slope estimate *S* of 0.000 (Table 48), whereas EDB demonstrated a higher mutagenic response (1.0103 rev/µg) than that reported in the literature (Table 47 (0.3 rev/µg). The increased mutagenic potential of EDB was present in the preincubation assay. Lower activity was demonstrated with EDB in both pour plate assays. The pour-log assay had an *S* of 0.3435 rev/µg. The pour-stationary assay had an *S* of 0.6632 rev/µg, which was similar to the activity demonstrated in Table 47. Rannug et al. (1978) demonstrated that these two compounds were activated through different mechanisms. EDB was a direct-acting mutagen, whose activity was increased by addition of phenobarbital-induced rat liver (125 µl S9/plate in 500 µl S9 mix) and NADPH with TA1535. Mutagenic activity of EDC required the addition of S9, but was independent of the mixed function oxidase system (i.e., activity did not require the addition of NADPH). The activation mechanism of EDC required glutathion conjugation with the proteins in the phenobarbital induced S9 from rats, with the subsequent formation of a mutagenic, highly reactive half-sulfur mustard (testing of EDB and EDC was not performed with S9 under this program). The study of Rannug et al. (1978) demonstrated that phenobarbital should be investigated as an inducer of S9 and compared with Aroclor-induced S9 with these vapor-phase compounds. Other investigators (Malaveille et al., 1977; Bartsch and Montesano, 1975; Garro et al., 1976; deMeester et al., 1980) employed phenobarbitone induction to detect the mutagenicity of the vapor-phase mutagen vinyl chloride.

The study of Rannug et al. (1978) also demonstrated that vapor-phase mutagens required large sample amounts to be detected. These workers tested EDC at 2,000, 4,000, and 6,000 μg/plate. Larger concentrations may be necessary due to the instability of vapor-phase compounds; high sample amounts also were required in this study. In addition, the vapor-phase results (Table 48) suggested that the statistical analysis systems of Myers et al. (1981) and Stead et al. (1981) would correctly predict known nonmutagens (EDC) as negative and known mutagens (EDB) as positive compounds.

A closely related compound to EDC and EDB was tested under this program (Table 48). This compound, ethylene oxide (ETO), was mutagenic in the preincubation assay (0.1453 rev/μg) and in the pour plate assay with stationary-phase cells (0.3675 rev/μg). The higher mutagenic activity in the pour plate stationary assay may have been due to an outlier in the spontaneous background rate which, when removed, reduced the activity to 1.780 rev/μg. Rannug et al. (1976) demonstrated that ETO was weakly mutagenic at 95.5 mM (4.4 μg/plate) with TA1535. Preincubation for one hr at 25°C was necessary to detect activity. In the preincubation validation work described within this report only TA100 was utilized because the addition of the R plasmid should increase the ability to detect mutagenic vapor-phase compounds (McCann et al., 1975). Rannug et al. (1976) also emphasized that lipophilic vapor-phase mutagens, such as vinyl chloride, have a low aqueous-solubility, and subsequently only a small fraction of the gaseous sample will react and be metabolized in the liquid state during preincubation. Consequently, methods should be investigated to solubilize vapors in the aqueous liquid preincubation media with organic solubilizers (Tween®) and of sonication.

The mutagenicity testing of butylene oxide with these three techniques (Section 5) demonstrated that the preincubation assay was superior to the pour plate assay, and that log-phase cells were more sensitive than stationary-phase cells. This was statistically confirmed by comparing the initial slope estimates (Table 48): preincubation with log-phase cells had an $\underline{S}$ of 0.4846 rev/μg; pour plate assay with log-phase cells had an $\underline{S}$ of 0.0336 rev/μg; and pour plate assay with stationary-phase cells had an $\underline{S}$ of 0.0972 rev/μg. Statistical analysis demonstrated that the preincubation technique with log-phase cells was approximately 15 times more sensitive in detecting the mutagenicity of butylene oxide than was the pour plate technique with log-phase cells. A large coefficient of variance was demonstrated for butylene oxide with the pour plate assay with log-phase cells (10.1666). This large variable suggests that butylene oxide may not be stable in the plate incorporation assay, or may not be as uniformly exposed as in the preincubation assay.

Propylene oxide produced results similar to butylene oxide. Mutagenic activity was superior with the preincubation technique by a factor of 10. However, propylene oxide was not toxic to the tester strains at the doses tested, whereas a large toxic potential was demonstrated with butylene oxide in the preincubation technique.

The mutagenicity of vinylidene chloride was statistically analyzed by the method of Myers et al. (1981) and activity with all three techniques was approximately equal (Table 48). Results with the preincubation and plate incorporation techniques with log-phase cells (0.88 rev/μg), but activity was considerably less with the plate incorporation and log-phase cells (0.41 rev/μg). Analysis of this data was difficult due to a high spontaneous back mutation rate, and high toxicity of vinylidine chloride to the bacteria. The weak mutagenic potential of this promutagen in the preincubation technique may be the result of an inappropriate generating system. Malaveille et al. (1977, 1975) have demonstrated the mutagenicity of vinyl chloride with phenobarbitone-induced mouse liver. In addition, base layer plates were incubated in a dessicator for 48 hr with 0.2, 2.0, and 20% v/v vinyl chloride, and an NADPH generating system was used. The protocol utilized in this EPA study was rat liver induced with Aroclor-1254; NADPH was added directly. Further validation with phenobarbital induced rat and mouse liver should be performed in conjunction with an NADPH generating system. Bartsch and Montesano (1975) demonstrated a negative mutagenic response for vinyl chloride in liquid suspension, which may be the result of technical difficulties associated with liquid suspension assays. In this study with the preincubation technique, a mutagenic ratio of approximately 4.0 was indicated at a dose level of 2,500 μg/plate (Table 44). This result compared favorably with the mutagenic potentials demonstrated in the review article of vinyl chloride by Bartsch and Montesano (1975). The experiments cited in the review article utilized large sample amounts (20% v/v) in dessicators and long incubation times (6-48 hr). The preincubation assay developed in this report required relatively small sample amounts (1,000-3,000 μg/plate) and an incubation time of only 30 min. This ability to detect small sample amounts is important in environmental testing of ambient air where sample is limited and long sampling times increase the possiblity of artifact generation.

The above results suggest that the preincubation technique with log-phase cells was the most sensitive technique for detection of vapor-phase organic mutagens. While results with some compounds were encouraging (styrene oxide, ethylene dibromide, ethylene oxide, propylene oxide, and butylene oxide), additional research is necessary to validate the sensitivity of this technique with other compounds (EDC and vinylidene chloride). For example, further tests could be conducted to compare S9 metabolic activation systems prepared from livers induced with phenobarbital and Aroclor. Increased amounts of S9 should also be evaluated for optimal promutagen activation. Rannug et al. (1978) utilized 125 μl of S9 per plate, whereas the normal amount is usually 50 μl of S9/plate (10%) (Ames et al., 1975b).

Two further variables that should be evaluated are NADPH and preincubation time. An NADPH generating system may in some cases (Malaveille et al., 1977, 1975) detect mutagens that the direct addition of NADPH would not. Preincubation time is also an important variable to be further evaluated, particularly if studies from different laboratories are to be compared. For example, Nagao et al. (1977) utilized incubation times of less than 20 min, while Yahagi et al. (1977) utilized preincubation times of up to 50 min. Further investigations into S9 stabilization with enzyme stabilizers such as EDTA with phenobarbital induced S9

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should be performed with the goal of extending preincubation time. Incubation time should be further evaluated with vapor-phase mutagens, since a nonvolatile compound (BAP) was utilized to determine preincubation time in this study (Figure 12). Epichlorohydrin which was not mutagenic in this study, but was mutagenic in the study by Bridges (1978). The inability to detect this compound may be due to preincubation time, since a 60 min preincubation time was utilized by Bridges and only 30 min was utilized in this study.

The preincubation technique developed under this program (Table 7) detected vapor-phase mutagens in a dose-response manner. The mutagenicity of these vapor-phase mutagens may have been detected at concentrations not found in ambient air. However, Bridges (1978) stated that many vapor-phase mutagens require from 2,000 to 4,000 μg/plate for detection. The method needs further validation with replicate samples of direct-acting and indirect acting vapor-phase mutagens and manipulations of the above-mentioned variables of preincubation time and NADPH-generating systems. For example, Yahagi et al. (1977) employed NADPH (2 μm), NADP (2 μm), ATP (2.5 μm), and three times the usual amount of S9 per plate (150 μl of S9/plate). These variations of the S9 metabolic activation system should be investigated. To improve the sensitivity of the assay, the variation of Guttenplan (1979) is promising. Guttenplan (1979) utilized pretreatment of bacterial tester strains with low amounts of vapors, and significantly increased the mutagen detection capability. Different solvents may also affect the ability of the preincubation assay to detect vapor-phase mutagens (Maron et al., 1981), and should be investigated.

In summary, the preincubation technique with log-phase cells was generally more sensitive to vapor-phase organic mutagens than the standard pour plate technique. This increased detection capability was statistically verified. The liquid suspension preincubation is suitable for the evaluation of vapor-phase ambient air field samples. The utilization of the preincubation assay, in tandem with GC/MS analysis of Tenax® trapped vapors, allows quantitative chemical and biological analysis of potentially mutagenic vapor-phase air samples.

Chemical Identification of Signature Mutagens and Carcinogens in VPO Field Samples

Table 50 lists the mutagens and carcinogens that were identified from vapor-phase field samples from four cities. These compounds are low molecular weight, chlorinated, short-chained compounds such as vinyl chloride and tetrachloroethylene, and compounds with high volatility, such as benzene. It should be noted that vapor-phase mutagens studied under this program (EDB and vinylidene chloride) were identified in ambient air.

MINIMAL BIOLOGICAL AND CHEMICAL TEST PROTOCOL FOR AMBIENT AIR

Table 51 outlines a minimal testing protocol for biological and chemical testing of ambient air particles (POM) and vapors (VPO). This

TABLE 50. COMPOUNDS IDENTIFIED BY GC/MS IN VAPOR-PHASE ORGANIC SAMPLES FROM 4 SITES[a] THAT ARE EITHER MUTAGENS (McCann et al., 1975) AND/OR CARCINOGENS (Sawicki, 1979)

Benzene (and benzene isomer)

Carbon tetrachloride

Chloroform

Ethylene dibromide

Napthalene

Phenol

Trichloroethane

Trichloroethylene

Tetrachloroethylene

Vinyl chloride

Vinylidene chloride

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[a] Sites sampled and tested were Lake Charles, Louisiana; Beaumont, Texas; Houston, Texas, and Elizabeth, New Jersey (Table 45 contains data by site).

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TABLE 51. OUTLINE OF SUGGESTED MINIMAL BIOLOGICAL AND CHEMICAL TEST PROTOCOL FOR AMBIENT AIR.

POM

1. Size-fractionation of ambient air particles; collection of a minimum of 30g of sample. Documented collection conditions.
2. Chemical fractionation of the crude organic extract into general chemical-classes; quantification of each chemical-class and total organic extract.
3. Chemical identification of signature mutagens. Quantification of active components in relation to amount of air sampled (rev/m^3).
4. Ames/Salmonella mutagenicity testing:
   - a. Toxicity prescreen at 2,000 μg/plate with TA98;
   - b. Minimum of five dose levels, with 2,000 μg extract/plate as the minimal highest dose depending on toxic potential (acceptable dose: >50% viability);
   - c. Triplicate plates with the plate incorporation technique;
   - d. Strain priority according to the following scheme: TA98, TA100, TA1535, TA1537, and TA1538;
   - e. Testing to be performed with one strain, at five dose levels, in triplicate, both with and without addition of Aroclor-induced rat S9. Initial testing should be completed with one strain before testing is started with the next strain;
   - f. Replicate dose response curves on active fractions;
   - g. Mutagenicity testing of both crude organic extracts and chemical-class fractions;
   - h. Vary S9 amount in repeat mutagenicity assay when mutagenic activity is detected with S9;
   - i. Replicate samples collected when mutagenic activity is detected.

VPO

1. Collection of ambient air vapor-phase organic (VPO) samples in tandem with particle sampling.
2. Collection of VPO on Tenax® cartridges.
3. Chemical identification of signature mutagens within one week after sampling. Cartridges must be kept at -80°C. Quantification of active components in relation to amount of air sampled (rev/m^3).
4. Thermal desorption/cryogenic freezing of samples for bioassay.
5. Ames/Salmonella mutagenicity testing:
   - a. Toxicity and mutagenicity testing conducted in tandem with the developed preincubation assay;
   - b. Strain priority: TA100, TA98, TA1535, TA1537, and TA1538;
   - c. Testing should be performed with one strain, at five dose levels, in triplicate, with and without Aroclor-induced S9. An NADPH-generating system should be utilized. Testing with one strain should be completed before testing is started with the next strain;
   - d. Initial doses should be greater than 5,000 μg/plate, and will depend on toxic potential;
   - e. Replicate dose responsive mutagenicity curves should be generated with active samples.

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protocol is the direct result of work conducted under this program. Specific procedures are explained in this report.

Additional research questions still need to be addressed, however. In addition to the large amount of particulate sample that is necessary for adequate initial testing, artifacts generated by sampling procedures and sample handling present problems. Pitts et al. (1979) have described experiments designed to define the generation of artifacts in sampling. Filters coated with 2 mg BAP were exposed to ambient air sampling for several days, and upon subsequent testing, direct mutagenicity was detected due to the formation of dihydrodiols, diphenols, phenols, quinones and nitro-metabolites of BAP. In controlled atmosphere experiments, when O_3 or NO_2 was reacted with BAP, direct-acting mutagens were also formed [hydroxybenzo(a)pyrene and 1,3- and 6-nitrobenzo(a)pyrenes]. Thus, promutagens can be converted to direct-acting mutagens by a mechanism not necessarily present in ambient air, but created during sampling. A more serious problem encountered was the formation of mutagens on the collection filter from two or more nonmutagens present in the air. For example, when perylene and NO_2 (both nonmutagens) were exposed in a controlled atmosphere, 3-nitroperylene, a potent mutagen, was formed. Consequently, artifact generation must be defined during extended sampling periods. The solvents used also may affect the detection of mutagenicity. Chrisp et al. (1978) have shown that mutagenicity was detected for industrial fly ash when horse serum was used as an extraction vehicle; Huisingh et al. (1979) have shown that diesel particulate is more mutagenic when Soxhlet extracted with dichloromethane than cyclohexane. These researchers also demonstrated a 50% loss of mutagenic activity and an increase in toxicity of direct-acting mutagens upon storage (2 months at 4°C).

There is an obvious lack of consistent extraction and assay protocols; some researchers use solvent extracts (Commoner et al., 1978; Flessel, 1977; Talcott and Wei, 1977) while others use complex extensive fractionation procedures (Teranishi et al., 1978; Tokiwa et al., 1977 and 1980). The fractionation scheme utilized in this study was developed by Pellizzari et al. (1979). Verification that chemicals were fractionated into their proper chemical-class after fractionation and that the fractionation scheme did not compromise the mutagenic potential of the sample (Section 4) was demonstrated.

The site of sample collection at major industrial complexes (Pellizzari, 1979) and time of year (Commoner et al., 1978) can make a difference in apparent mutagenicity. The use of a sampler that will divide the particulate according to size is recommended, as the smaller particulates (<1.7 μm) have been found to contain the greatest amount of mutagenic activity/unit sample mass. Particulate extracts should be fractionated into chemical-classes after solvent extraction in order to aid both chemical identification and detection of mutagenic activity. An integrated chemical and biological approach to the assessment of air pollutant hazard offers advantages that neither alone can provide.

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Further validation of the preincubation for vapor detection, which was developed under this program, should be performed to evaluate the sensitivity of the S9 system with regards to the amount of S9 protein and type of NADPH generating system, the optimal incubation time and the maximum storage time for vapor samples. Field studies should be performed with split samples and compared to laboratory studies. The development of a fully validated method to measure the mutagenicity of vapors in the Ames system will allow a reasonable comparison of mutagenic potential of vapors and particles.

Collection, fractionation and bioassay of ambient air samples should be optimized for sensitivity, accuracy and reproducibility of results. Additional controlled atmosphere studies will help define both artifact generation and comutagenic/antagonistic reactions which occur in the complex mixtures typical of polluted air. Permanent monitoring stations, established in areas where pollution is high, would help to define seasonal mutagenic fluctuations, and thus provide information on the influence of weather conditions and industrial output on the mutagenic potential air pollutants. Continued research on the influence of solvent extraction and chemical fractionation schemes on the mutagenicity of air particulate are recommended.

Particular attention should be directed towards further development of bioassay methods for quantitative vapor detection. The development of new methods and refinement and validation of existing bioassay methods should be part of a combined chemical-biological effort, since many variables (in both the chemical preparation of the sample and bioassay testing) can affect the mutagenic potential. These variables should be reduced to a minimum. A continued effort would emphasize quantitative information of mutagenic potential, expressed relative to the volume of air and particles sampled.

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SECTION 7

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APPENDIX A

METHODS FOR SAMPLING AND CHEMICAL ANALYSIS OF VAPOR-PHASE ORGANICS IN AMBIENT AIR

Principle of Method

Volatile organic compounds were concentrated from ambient air onto Tenax® GC sorbent in a short glass tube (1-3). Recovery of the volatile organics was accomplished by thermal desorption and purging with helium into a liquid nitrogen cooled nickel capillary trap (1,2,4) and then the vapors were introduced into a high resolution glass gas chromatographic column where the constituents were separated from each other (2,5). Characterization and quantification of the constituents in the sample were accomplished by mass spectrometry either by measuring the intensity of the total ion current signal or by mass fragmentography (2,6).

Range and Sensitivity

The linear range for the analysis of volatile organic compounds depends upon two principal features. The first is a function of the breakthrough volume of each specific compound which was trapped on the Tenax® GC sampling cartridge and the second is related to the inherent sensitivity of the mass spectrometer for each organic (2,7). Thus, the range and sensitivity are a direct function of each compound which is present in the original ambient air. The linear range for quantitation on the gas chromatograph/mass spectrometer/computer (GC/MS/Computer) was generally three orders of magnitude. Table A1 lists the overall theoretical sensitivity for some examples of volatile organics which is based on these two principles (7).

Interferences

The potential difficulties with this technique involved isomeric forms of a particular substance (which could not be resolved by the high resolution chromatographic column) and interpretation of identical mass spectral fragmentation patterns of these isomers. An example of such a problem was the C_5-alkyl aromatics, of which there are 53 isomers. As the number of carbon atoms increases in the hydrocarbons and aromatics, the number of potential isomers becomes increasingly large and difficult to completely resolve by gas chromatography and/or by their corresponding mass spectral fragmentation patterns. However, differentiation between hydrocarbons, (alkanes, alkenes, aromatics, oxygenated) can be accomplished.

TABLE A1. OVERALL THEORETICAL SENSITIVITY OF HIGH RESOLUTION GAS CHROMATOGRAPHY/MASS SPECTROMETRY/COMPUTER ANALYSIS FOR ATMOSPHERIC POLLUTANTS

Chemical Class	Compound	Estimated Detection Limit[a]	
		ng/m^3	ppt
Halogenated hydrocarbon	Vinyl bromide	250	57
	Bromoform	0.340	0.03
	Bromodichloromethane	1.300	0.22
	Dibromochloromethane	0.667	0.07
	1-Bromo-2-chloroethane	1.00	0.67
	Allyl bromide	5.00	1.04
	1-Bromopropane	5.200	1.06
	1-Chloro-3-bromopropane	0.150	0.01
	1-Chloro-2,3-dibromopropane	~0.100	<0.01
	1,1-Dibromo-2-chloropropane	~0.100	<0.01
	1,2-Dibromoethane	0.530	0.07
	1,3-Dibromopropane	~0.100	~0.01
	Epichlorohydrin (1-Chloro-2,3-epoxypropane)	9.600	2.50
	Epibromohydrin (1-Bromo-2,3-epoxypropane)	0.300	0.05
	Bromobenzene	0.100	0.02
	Methyl bromide	500	135
	Methyl chloride	2000	1000
	Vinyl chloride	800	333
	Methylene chloride	700	200
	Chloroform	200	420
	Carbon tetrachloride	250	400

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TABLE A1 (cont'd)

Chemical Class	Compound	Estimated Detection Limit[a]	
		ng/m³	ppt
Halogenated hydrocarbon (cont'd)	1,2-Dichloroethane	32	8.15
	1,1,1-Trichloroethane	66	12.45
	Tetrachloroethylene	2.5	0.38
	Trichloroethylene	10	1.92
	1-Chloro-2-methylpropene	62	21.5
	3-Chloro-2-methylpropene	62	21.5
	3-Chloro-1-butene	83	28.8
	Allyl chloride	83	28.8
	4-Chloro-1-butene	38	13.2
	1-Chloro-2-butene	13	4.5
	Chlorobenzene	2.10	0.47
	o-Dichlorobenzene	1.00	0.06
	m-Dichlorobenzene	0.75	0.01
	Benzylchloride	0.65	0.01
Halogenated ethers	2-Chloroethyl ethyl ether	4.15	0.97
	Bis-(chloromethyl)ether	1.0	1.10
Nitrosamines	N-Nitrosodimethylamine	5.0	1.67
	N-Nitrosodiethylamine	3.0	0.74
Oxygenated hydrocarbons	Acrolein	~100	56.5
	Glycidaldehyde	~59	9.5
	Propylene oxide	~60	25.5

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TABLE A1 (cont'd)

Chemical Class	Compound	Estimated Detection Limit[a]	
		ng/m^3	ppt
Oxygenated hydrocarbons (cont'd)	Butadiene diepoxide	~20	6.7
	Cyclohexene oxide	~10	2.5
	Styrene oxide	2	0.415
	Acetophenone	~2	~0.415
	β-Propiolactone	~3	~1.2
Nitrogenous Compounds	Nitromethane	8	~2.4
	Aniline	3.0	0.78
Sulfur Compounds	Diethyl sulfate	~50	-
	Ethyl methane sulfate	~5.0	-

[a] Limits are calculated on the basis of the breakthrough volume for 2.2 g of Tenax GC, (at 70°F), capillary column performance and sensitivity of the mass spectrometer to that compound in the mass fragmentography mode of most intense ion.

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Reproducibility

The reproducibility of this method has been determined to range from ±10-30% of the relative standard deviation for different substances when replicate sampling cartridges were examined (5). The inherent analytical errors were a function of several factors: [1] the ability to accurately determine the breakthrough volume for each of the organic compounds identified; [2] the accurate measurement of the ambient air volume sampled; [3] the percent recovery of the organic from the sampling cartridge after a period of storage; [4] the reproducibility of thermal desorption for a compound from the cartridge and its introduction into the analytical system; [5] the accuracy of determining the relative molar response ratios between the identified substance and the external standard used for calibrating the analytical system; [6] the reproducibility of transmitting the sample through the high resolution gas chromatographic column; and [7] the day-to-day reliability of the MS/Computer system (1-8).

The accuracy of analysis was generally ±30%; this value depended on the chemical and physical nature of the compound (2,8).

Advantages and Disadvantages of the Method

The gas chromatograph/mass spectrometer interfaced with a jet separator which was extremely sensitive and specific for the analysis of many volatile organic compounds found in ambient air. High resolution gas chromatographic separation provided adequate resolution of the substances found in ambient air. The combination of the high resolution gas chromatographic column and the selection of specific or unique ions representing the various compounds of interest identified in the air samples yielded a relatively specific assay method for these compounds (1-8).

Collected samples can be stored up to one month with less than 10% loss for most of the chemical classes (2,8). Because some of the compounds of interest may be hazardous to man, it is extremely important to exercise safety precautions in the preparation and disposal of liquid and gas standards, cleaning of used glassware, etc. in the analysis of air samples.

Since the mass spectrometer cannot be conveniently mobilized, sampling must be carried out away from the instrument.

The efficiency of air sampling increased as the ambient air temperature decreases (i.e., sensitivity increased) (8). The retention of water by Tenax® was low; its thermal stability was high and its background was small, which allowed sensitive analysis to be accomplished (1,2,5,8).

Reagents and Materials

All reagents used were of analytical reagent grade. All glassware, sampling tubes, cartridge holders, etc. were washed in Isoclean/water, rinsed with deionized distilled water and acetone, then air dried. Glassware was heated to 450-500°C for 2 hr to insure that all organic material was removed prior to use.

Preparation of Tenax® GC

Virgin Tenax® GC was extracted in a Soxhlet apparatus for a minimum of 18 hr with acetone or methanol prior to use. The Tenax® GC sorbent was dried in a vacuum oven at 100°C for 3-5 hr and then sieved to provide a fraction corresponding to 35/60 mesh. This fraction was used for preparing sampling cartridges. In those cases where sampling cartridges of Tenax GC were recycled, the sorbent was extracted in a Soxhlet apparatus with acetone or methanol as described for the virgin material, but the sorbent was further extracted with a nonpolar solvent, *n*-pentane, in order to remove the relatively nonpolar and nonvolatile materials which might have accumulated on the sorbent bed during previous sampling periods.

For large sample volumes, it was important that the total volume of air did not cause the elution of compounds through the sampling tube (*i.e.*, their breakthrough volume was not exceeded). The breakthrough volumes of some of the volatile organics are shown in Table A2 (2,4,8). These breakthrough volumes have been determined by a previously described technique (2). The breakthrough volume was defined as that point at which 50% of a discrete sample introduced into the cartridge was lost. Although the identity of a compound during ambient air sampling was not known, the compound can still be quantified after identification by GC/MS/Computer once the breakthrough volume has subsequently been established. Thus, for calculating concentration, the last portion of the sampling period which represents the volume of air sampled prior to breakthrough was selected. For cases in which the identity of a volatile organic compound was not known until after GC/MS, the breakthrough volume was subsequently determined.

Previous experiments have shown that the organic vapors collected on Tenax® GC sorbent were stable and could be quantitatively recovered from the cartridge samplers up to four weeks after sampling when they were tightly closed in cartridge holders and placed in a second container that was sealed, protected from light and stored at 0°C (1,2).

Analysis of Samples

The instrumental conditions for the analysis of volatile organics on the sorbent Tenax® GC sampling cartridge is shown in Table A3. The thermal desorption chamber and the six port Valco valve were maintained at 270° and 240°, respectively. The jet separator was maintained at 240°. The mass spectrometer was set to scan the mass range from approximately 20-350. The helium purge gas for the desorption chamber was adjusted to 15-20 ml/min. The nickel capillary trap on the inlet manifold was cooled with liquid nitrogen. In a typical thermal desorption cycle, a sampling cartridge was placed in the preheated desorption chamber and the helium gas was channeled through the cartridge to purge the vapors

TABLE A2. TENAX® GC BREAKTHROUGH VOLUMES FOR SEVERAL ATMOSPHERIC POLLUTANTS[1]

Chemical Class	Compound	b.p. (°C)	Temperature (°F) 50	60	70	80	90	100
Halogenated hydrocarbon	methyl chloride	-24	8	6	5	4	3	2.5
	methyl bromide	3.5	3	2	2	1	1	0.9
	vinyl chloride	13	2	1.5	1.25	1.0	0.8	0.6
	methylene chloride	41	11	9	7	5	4	3
	chloroform	61	42	31	24	18	13	10
	carbon tetrachloride	77	34	27	21	16	13	10
	1,2-dichloroethane	83	53	41	31	23	18	14
	1,1,1-trichloroethane	75	23	18	15	12	9	7
	tetrachloroethylene	121	361	267	196	144	106	78
	trichloroethylene	87	90	67	50	38	28	21
	1-chloro-2-methylpropene	68	26	20	16	12	9	7
	3-chloro-2-methylpropene	72	29	22	17	13	10	8
	1,2-dichloropropane	95	229	162	115	81	58	41
	1,3-dichloropropane	121	348	253	184	134	97	70
	epichlorohydrin (1-chloro-2,3-epoxypropane)	116	200	144	104	74	54	39
	3-chloro-1-butene	64	19	15	12	9	7	6
	allyl chloride	45	21	16	12	9	6	5
	4-chloro-1-butene	75	47	36	27	20	15	12
	1-chloro-2-butene	84	146	106	77	56	40	29
	chlorobenzene	132	899	653	473	344	249	181
	o-dichlorobenzene	181	1,531	1,153	867	656	494	372
	m-dichlorobenzene	173	2,393	1,758	1,291	948	697	510

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Chemical Class	Compound	b.p. (°C)	Temperature (°F) 50	60	70	80	90	100
Halogenated hydrocarbons (cont'd)	**benzyl chloride**	179	2,792	2,061	1,520	1,125	830	612
	bromoform	149	507	386	294	224	171	131
	ethylene dibromide	131	348	255	188	138	101	74
	bromobenzene	155	2,144	1,521	1,079	764	542	384
Halogenated Ethers	**2-chloroethyl ethyl ether**	108	468	336	241	234	124	89
	Bis-(chloromethyl)ether	-	995	674	456	309	209	142
Nitrosamines	**N-nitrosodimethylamine**	151	385	280	204	163	148	107
	N-nitrosodiethylamine	177	2,529	1,836	1,330	966	700	508
Oxygenated hydrocarbons	**acrolein**	53	19	14	10	8	6	4
	glycidaldehyde	-	364	247	168	114	77	52
	propylene oxide	34	35	24	17	11	8	5
	butadiene diepoxide	-	1,426	1,009	714	506	358	253
	cyclohexene oxide	132	2,339	1,644	1,153	811	570	400
	styrene oxide	194	5,370	3,926	2,870	2,094	1,531	1,119
	phenol	183	2,071	1,490	1,072	769	554	398
	acetopheonone	202	3,191	2,382	1,778	1,327	991	740
	β-propiolactone	57	721	514	366	261	186	132
Nitrogenous Hydrocarbons	**nitromethane**	101	45	34	25	19	14	11
	aniline	184	3,864	2,831	2,075	1,520	1,114	817
Sulfur Compounds	**diethyl sulfate**	208	40	29	21	15	11	8
	ethyl methane sulfate	86	5,093	3,681	2,564	1,914	1,384	998

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TABLE A2 (cont'd)

Chemical Class	Compound	b.p. (°C)	Temperature (°F) 50	60	70	80	90	100
Amines	dimethylamine	7.4	9	6	4	3	2	1
	isobutylamine	69	71	47	34	23	16	11
	t-butylamine	89	6	5	4	3	2	1
	di-(n-butyl)amine	159	9,506	7,096	4,775	3,105	2,168	1,462
	pyridine	115	378	267	189	134	95	67
	aniline	184	8,128	5,559	3,793	2,588	1,766	1,205
Ethers	diethyl ether	34.6	29	21	15	11	8	5
	propylene oxide	35	13	9	7	5	4	3
Esters	ethyl acetate	77	162	108	72	48	32	22
	methyl acrylate	80	164	111	75	50	34	23
	methyl methacrylate	100	736	484	318	209	137	90
Ketones	acetone	56	25	17	12	8	6	4
	methyl ethyl ketone	80-2	82	57	39	27	19	13
	methyl vinyl ketone	81	84	58	40	28	19	14
	acetophenone	202	5,346	3,855	2,767	2,000	1,439	1,037
Aldehydes	acetaldehyde	20	3	2	2	1	0.9	0.7
	benzaldehyde	179	7,586	5,152	3,507	2,382	1,622	1,101
Alcohols	methanol	64.7	1	1	0.8	0.6	0.4	0.3
	n-propanol	97.4	27	20	14	10	7	5
	allyl alcohol	97	32	23	16	11	8	6

(continued)

TABLE A2 (cont'd)

Chemical Class	Compound	b.p. (°C)	Temperature (°F)					
			50	60	70	80	90	100
Aromatics	benzene	80.1	108	77	54	38	27	19
	toluene	110.6	494	348	245	173	122	86
	ethylbenzene	136.2	1,393	984	693	487	344	243
	cumene	152.4	3,076	2,163	1,525	1,067	750	527
Hydrocarbons	n-hexane	68.7	32	23	17	12	9	6
	n-heptane	98.4	143	104	75	55	39	29
	1-hexene	63.5	28	20	15	11	8	6
	1-heptene	93.6	286	196	135	93	64	44
	2,2-dimethylbutane	49.7	0.5	0.4	0.3	0.2	0.2	0.1
	2,4-dimethylpentane	80.5	435	252	146	84	49	28
	4-methyl-1-pentene	53.8	14	10	8	6	4	3
	cyclohexane	80.7	49	36	26	19	14	10
Inorganic gases	nitric oxide	-	0	0	0	0	0	0
	nitrogen dioxide	-	0	0	0	0	0	0
	chlorine	-	0	0	0	0	0	0
	sulfur dioxide	-	0.06	0.05	0.03	0.02	0.02	0.01
	water	100	0.06	0.05	0.04	0.03	0.01	0

[a] Breakthrough volume is given in ℓ/2.2 g Tenax GC used in sampling cartridges.

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TABLE A3. OPERATING PARAMETERS FOR GC/MS COMP SYSTEM

Parameter	Setting
Inlet-manifold	
desorption chamber and valve	270°C
capillary trap - minimum	-195°C
maximum	240°C
thermal desorption time	8 min
He purge flow	15 ml/min
GLC	
100 m glass SCOT SE-30	25-240°C, 4°C/min
carrier (He) flow	~3 ml/min
MS	
Varian MAT CH-7	
scan range	*m/z* 20 → 350
scan cycle, automatic-cyclic	1 sec/decade
filament current	300 μA
multiplier	4.0
ion source vacuum	$\sim 4 \times 10^{-6}$ torr
Finnigan 3300	
scan range	*m/z* 20 → 350
scan cycle, automatic-cyclic	2 sec/cycle
filament current	0.50 mA
multiplier	1.7 kV
ion source vacuum	$\sim 1 \times 10^{-5}$ torr
LKB 2091	
mass range	*m/z* 5 → 492
scan cycle, automatic-cyclic	2.4 sec/cycle (parabolic scan)
filament current	4A
multiplier	300
ion source vacuum	3×10^{-6} torr

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into the liquid nitrogen capillary trap [the inert activity of the trap has been shown in a previous study (5)]. After the desorption has been completed, the six-port valve was rotated and the temperature on the capillary loop was rapidly raised (greater than 10°/min); the carrier gas then introduced the vapors onto the high resolution GC column. The glass capillary column was temperature programmed from ambient to 240°C at 4°C/min and held at the upper limit for a minimum of 10 min. After all the components have eluted the column was then cooled to ambient temperature and the next sample is processed (2).

An example of the analysis of volatile organics in ambient air is shown in Figure A3 and the background from a blank cartridge is shown in Figure A4. The high resolution glass capillary column was coated with SE-30 stationary-phase which was capable of resolving a multitude of compounds to allow their subsequent identification by MS/COMP techniques.

Operation of the MS/COMP System (Figure A5)

The Varian MAT CH-7 mass spectrometer was first set to operate in the repetitive scanning mode. In this mode the magnet was automatically scanned exponentially upward from a preset low mass to a high mass value. Although the scan range may be varied depending on the particular sample, typically the range was set from approximately 1.8 sec. At this time the instrument automatically reset itself to the low mass position in preparation for the next scan, and the information was accumulated by an on-line 620/L computer and written onto magnetic tapes. The reset period required approximately 2.0 sec. Thus, a continuous scan cycle of 3.8 sec/scan was maintained and repetitively executed throughout the chromatographic run. The result was the accumulation of a continuous series of mass spectra throughout the chromatographic run in sequential fashion.

Prior to analysis of unknown samples, the system was calibrated by introducing a standard substance, perfluorokerosene, into the instrument and determining the time of appearance of the known standard peaks in relation to the scanning magnetic field. The calibration curve thus generated was stored in the 620/L computer memory. This calibration served to calibrate the ions over the scanning range.

While the magnet was continuously scanning, the sample was injected and automatic data acquisition was initiated. As each spectrum was acquired by the computer, each peak which exceeded a preset threshold was recognized and reduced to centroid time and peak intensity. This information was stored in the computer core while the scan was in progress. In addition, approximately 30 total ion current values and an equal number of Hall probe signals were stored in the core of the computer as they were acquired. During the two second period between scans, this spectral information, and spectrum number was written sequentially on magnetic tape and the computer was reset for the acquisition of the next spectrum.

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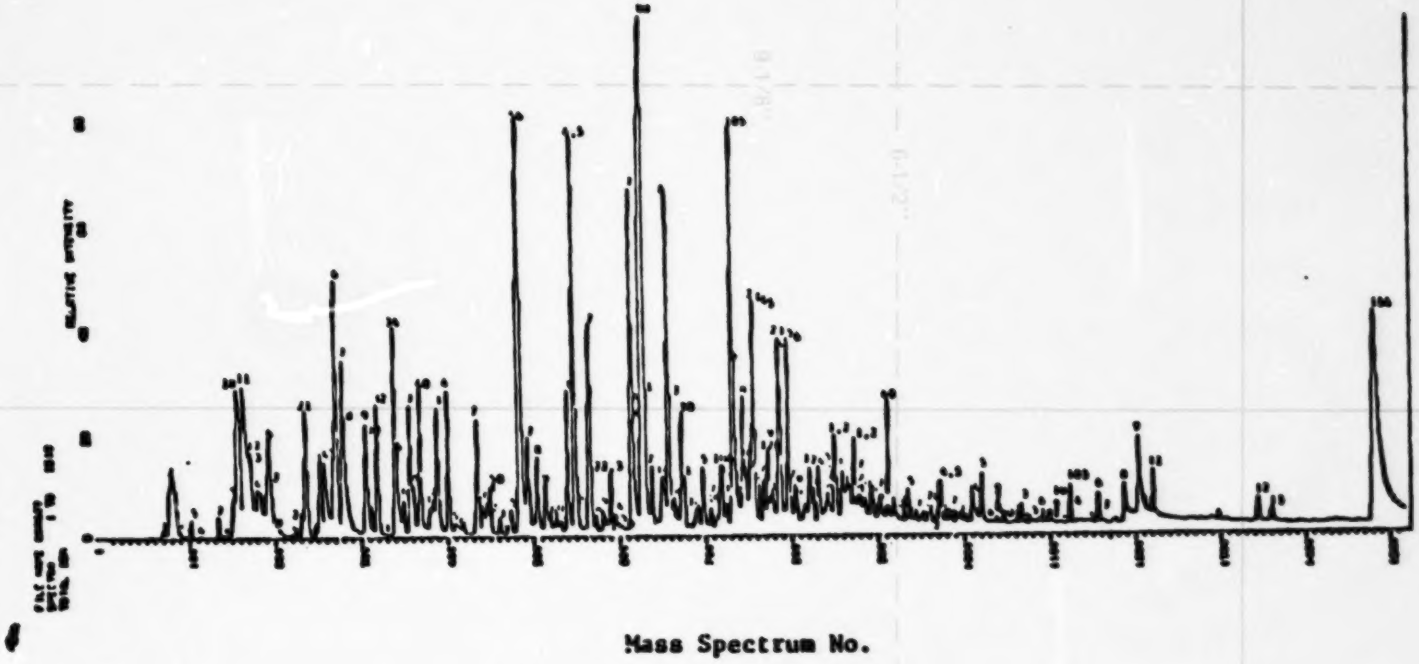

Figure A3. Profile of ambient air pollutants obtained using high resolution gas chromatography/ mass spectrometry/computer.

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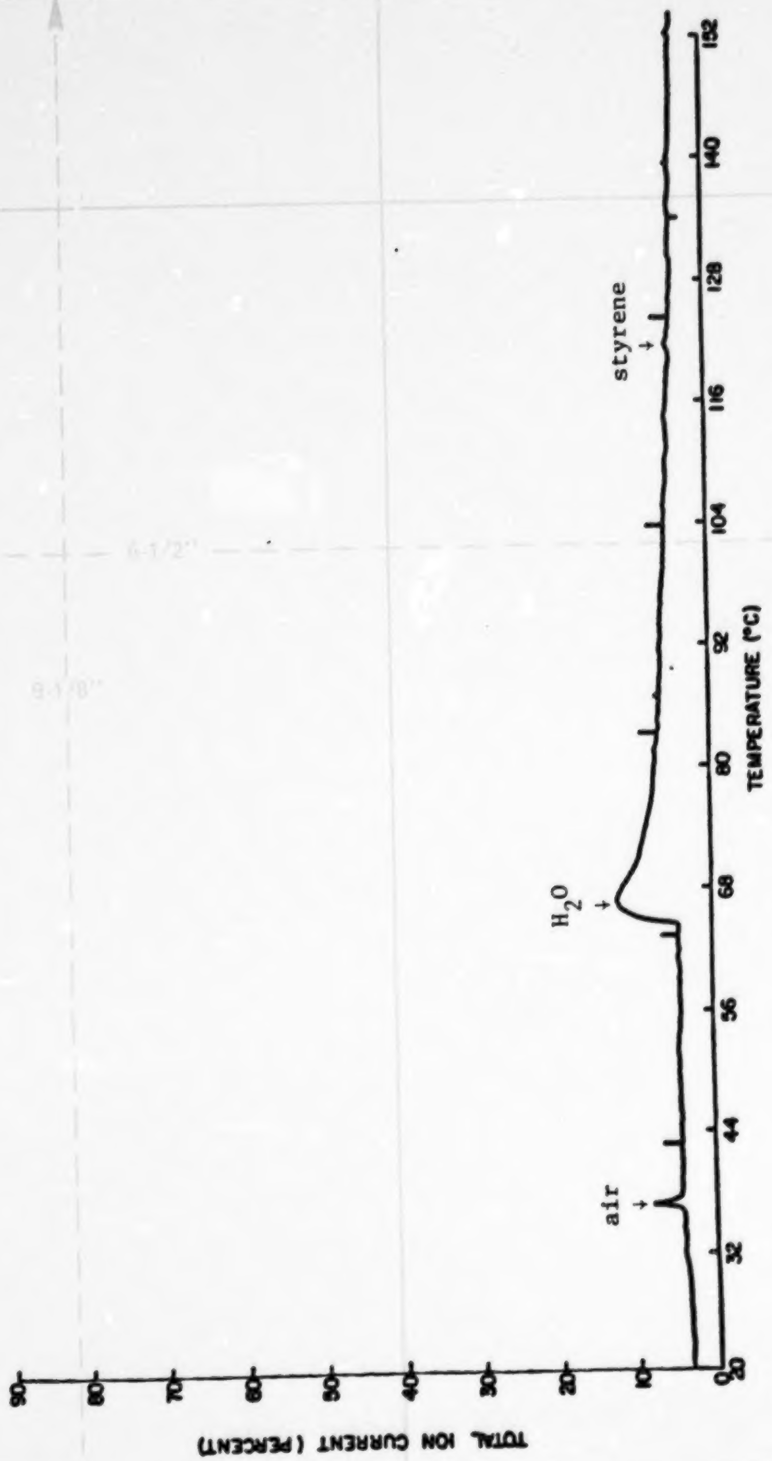

Figure A4. Background profile for Tenax® GC cartridge blank.

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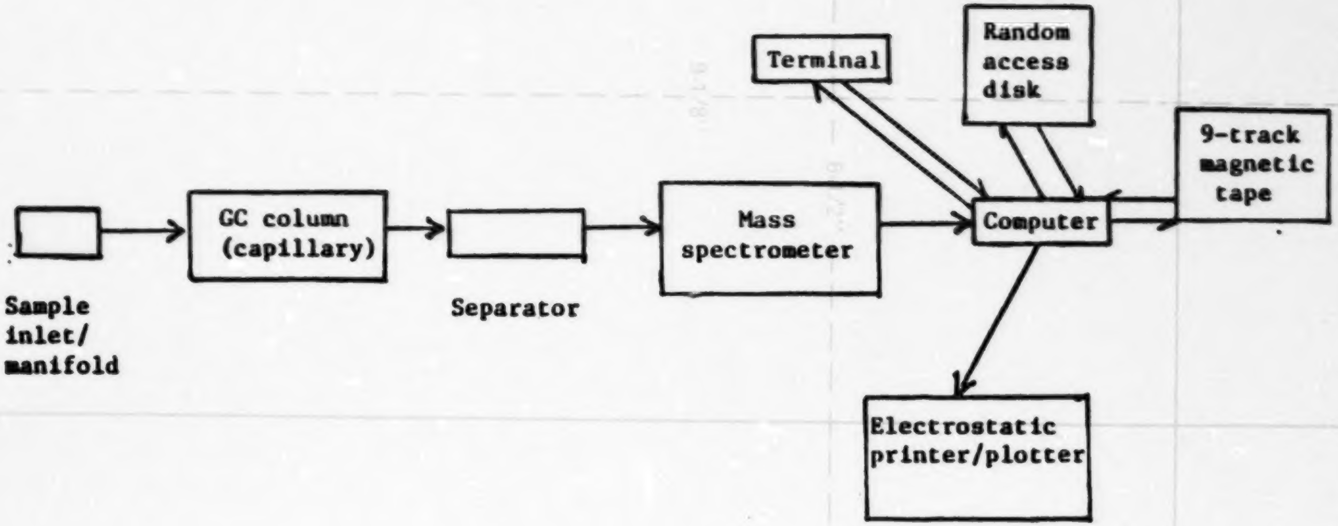
Sample
inlet/
manifold
GC column
(capillary)
Separator
Mass
spectrometer
Terminal
Random
access
disk
Computer
9-track
magnetic
tape
Electrostatic
printer/plotter

Figure A5. Schematic diagram of GC/MS/Computer system.

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This procedure continued until the entire GC run was completed. Depending on the information required, this information may then either be processed immediately or additional samples may be run, stored on magnetic tape and the results examined at a later time.

The mass spectral data were processed in the following manner. First, the original spectra were scanned and the total ion current (TIC) information was extracted. The TIC intensities were plotted against the spectrum number on the Statos 31 recorder. This information indicated whether the run was suitable for further processing, since it provided some idea of the number of unknowns in the sample and the resolution obtained using the particular GC column conditions.

The next stage of the processing involved the conversion of the spectra; peak times to peak masses was done directly via the dual disk system. The mass conversion was accomplished by use of the calibration table obtained previously with perfluorokerosene. Normally one set of the calibration data was sufficient for an entire day's data processing since the characteristics of the Hall probe are such that the variation in calibration was less than 0.2 daltons/day. A typical time required for this conversion process for 1,000 spectra was approximately 30 min.

After the spectra were obtained in mass converted form, processing proceeded either manually or by computer. In the manual mode, the full spectrum of scans for the GC run was recorded on the Statos 31 plotter. The TIC information available at this time was useful for deciding which spectra should be analyzed. At the beginning of the runs where peaks were very sharp, nearly every spectrum must be inspected individually to determine the identity of the component. Later in the chromatographic run when the peaks are broader only selected scans were analyzed.

The Finnigan 3300 mass spectrometer was set to operate in the repetitive scanning mode, scanning the quadrupole from low mass to high mass. A typical range was from $\underline{m}/\underline{z}$ 20 to $\underline{m}/\underline{z}$ 350. The scan required approximately 1 sec; the instrument then rests at the high end of the mass scale and then automatically re-initiated a scan cycle at the low mass. During the rest/reset period, data from the previous scan were accumulated by an on-line PDP-12 computer and written on disk. The scan cycle of 2.0 sec was repetitively executed throughout the chromatographic run, resulting in the accumulation of a continuous series of mass spectra.

The quadrupole mass spectrometer acquired data as masses and intensities. A calibration table was obtained by use of perfluorotributylamine (FC-43) to correlate voltages and masses. Normally one set of calibration data sufficed for a day of data acquisition. The data was then presented as TIC information (obtained by summing ion intensities over the entire spectrum) with chromatographic peak intensities plotted against time.

The LKB 2091 mass spectrometer was operated under computer control. The start time and scan interval time were selected by the operator. The magnet was scanned from $\underline{m}/\underline{z}$ 5 to $\underline{m}/\underline{z}$ 490 at a scan speed of 2 sec full scan (0-670 daltons). The scan cycle time was typically 2.4 sec. Mass *vs*. intensity data was acquired by the computer and stored on disk. The mass values were determined by the LKB mass marker and modified as necessary by a computer program which corrected for deviations due to scan speed. The mass calibration tables were stable for over a six month period of time. The data was then plotted as total ion current (over an operator determined mass range usually *m/z* 33 to *m/z* 492) versus spectrum number. This information was useful in deciding which spectra should be plotted and interpreted. Normally all spectra from the air peak on are plotted. Data was stored archivally on 9-track IBM compatible magnetic tapes.

Identification of resolved components from data produced by all the mass spectrometer systems was achieved by comparing the fragmentation patterns of the unknown mass spectra to an eight major peak index of mass spectra (9). Individual difficult unknowns were searched by the use of the Cornell University STIRS and PBM systems. Unknowns were also submitted to MSSS system for identification. When feasible, the identification of unknowns was confirmed by comparing the fragmentation pattern and elution temperatures for two different chromatographic columns (SE-30 and Carbowax SCOT capillaries) for the unknown and authentic compounds. The relationship between the boiling point of the identified compound and the elution temperature on a nonpolar column (the order of elution of constituents was predictable in homologous series since the SE-30 SCOT capillary separated primarily on the basis of boiling point) was carefully considered in making structure assignments.

Mass spectral search programs are operational at the Triangle Universities Computation Center (TUCC). RTI maintains twice daily service to TUCC, which is a one-quarter mile distance from the RTI campus. Additional information about each magnetic tape containing mass spectra was entered directly into TUCC with a remote job entry processing. This was performed by TUCC via one of the five terminals located within the Analytical Sciences Laboratory. The control information contained selected spectrum numbers with instructions to process entire GC runs. The computer program systems simultaneously compared either the entire library of 25,000 compounds or some subset of this library. The completed reports demonstrating the best fits for each of the unknowns were produced at TUCC and printed out at the high speed terminals located on the RTI campus or TUCC.

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APPENDIX B

PROTOCOL FOR THE AMES/*SALMONELLA* MUTAGENICITY ASSAY

The major objective of this contract was to apply the *Salmonella*/mammalian-microsome mutagenicity test to the evaluation of air samples, both as complex mixtures and after fractionation. The mutagenicity tests were conducted in conjunction with analytical chemical fractionation techniques. These techniques separate the crude sample using solvents compatible with the bioassay, and identify materials likely to represent presumptive mutagens and carcinogens, based on their chemical properties.

The mutagenicity test procedures were based on those developed by Ames et al. (1975). Certain modifications were adopted as follows:

(1) Use of NADPH in lieu of a NADPH-generating system (Frantz and Malling, 1975; Yahagi et al., 1975);

(2) Use of Craig-Dawley strain of rats, rather than Sprague-Dawley strain (recommended by project officers, Drs. Larry Claxton and Michael Waters, in order for the S9 protein to conform to EPA in-house research);

(3) Use of 0.25 M sucrose solution, rather than KCl solution, in preparation of S9 (Frantz and Malling, 1975).

NADPH was utilized at 320 µg/plate in the Ames assay [3.2 mg of NADPH (Sigma - #N1630) was dissolved in 1.0 of sterile deionized-distilled water and 100 µl of this solution was added per plate]. The use of NADPH, instead of an NADPH-generating system, has been utilized with the Ames/*Salmonella* assay in liquid culture (Frantz and Malling, 1975). NADPH was suggested for use in the pour plate assay by these researchers as a means to avoid the generation of false negative results by highly toxic air samples. Toxic samples could inhibit the NADPH-generating system and therefore cause false negative results.

The results with NADPH at RTI have produced similar results to those published by McCann et al. (1975) in their validation paper with 300 chemicals. NADPH is used extensively in Japan (instead of a NADPH-generating system), at the National Cancer Center Research Institute (Yahagi et al., 1975, 1977; Nagao et al., 1977). NADPH is used extensively in Japan because it is inexpensive there (Ames, 1980). However, the Japanese results compare favorable with those of Dr. Ames' group at Berkeley (Ames, 1980).

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Craig-Dawley (CD) and Sprague-Dawley (SD) male rats were compared as sources of liver S9. In preliminary tests, Aroclor 1254-induced S9 preparations of 20 mg protein/ml were prepared. The tester strain was TA98 and the known mutagens employed were benzo(a)pyrene, 2-acetylaminofluorene, 2-anthramine, and 2-nitrofluorene. Results of the tests are shown in Table B1. Overall differences between the two strains were marginal. However, since the microsomal preparations were not made at the same time and the rats were not the same size and age, the tests were repeated on a larger scale.

In these tests, strain TA98 was used; known mutagens used were benzo(a)pyrene, 2-acetylaminofluorene, 2- anthramine, and 20-methylchlanthrene. The amount of S9 per plate was also varied. Results are shown in Figures B1-B3. In general, results with the two rat strains were comparable, and in the case of 2-acetylaminofluorene, were strikingly similar. Obviously, a definitive comparison of the two strains would require much more extensive testing with all five *Salmonella typhimurium* strains and a larger variety of test chemicals. Such comparison was beyond the scope of this project. It was therefore concluded from these experiments that activation differences between the two rat strains were negligible, and that Craig-Dawley rat livers would be utilized as the S9 source in this testing.

Salmonella typhimurium strains utilized in this study were the histidine auxotrophs able to detect frameshift mutations (TA98, TA1537, TA1538) and base-pair substitution mutations (TA100, TA1535) which were developed by Dr. Bruce Ames at Berkeley, California (Ames et al., 1975). Reversion to wild type (histidine independence) upon addition of sample was the mutagenic marker. Mutagenicity was directly proportional to the number of mutant colonies produced upon addition of sample. All strains were obtained from Bruce N. Ames, University of California at Berkeley. Metabolic activation was achieved by addition of S9 microsomal preparation from the liver of male rats, Craig-Dawley strain; induction of liver enzymes was accomplished by a single peritoneal injection of Aroclor 1254, in corn oil, five days prior to sacrifice. Incubation was at 37°C for 48-72 hr. Colonies were counted with a Fisher® automatic colony counter (Count-al); plates with less than 100 colonies were hand counted.

Preparation of S9 from Rat Liver Microsomes--The procedure of Ames et al. (1975), was used.

Male Charles River rats, strain CD_1 (200 g ± 20 g) served as the source of liver material. The animals were housed one week before initiation of induction in suspended cages. Food was removed from the animal 12 h before sacrifice. Water was given *ad libitum*. Induced rat liver (minimum of three rats) was obtained from rats injected intraperitoneally on day one with 500 mg/kg of Aroclor 1254 in Mazola® Corn Oil (0.5 ml of a 200 mg/ml for a 200 g rat). On day five, the animals were sacrificed with a guillotine. The liver was removed and placed in a sterile, preweighed beaker. Using sterile technique, livers were immersed in cold, sterile 0.25 *M* sucrose, washed two times, and blotted dry with sterile paper (Kimwipes®). The liver was weighed, minced, and brought

TABLE B1. COMPARISON OF CRAIG-DAWLEY (CD) RATS WITH SPRAGUE-DAWLEY (SD) RATS AS SOURCE OF INDUCED LIVER S9[a]

Test Compound	Concentration µg/plate	No. Colonies Produced (Avg. = 3 Plates) No. S-9	S-9 CD	S-9 SD	Relative Mutagenesis Ratio CD	Relative Mutagenesis Ratio SD
benzo(a)pyrene	100	63	201	206	3.2^m	3.3^m
	10	65	187	166	2.9^m	2.6^m
	5	65	170	219	2.6^m	3.4^m
	1	50	134	201	2.7^m	4.0^m
2-acetylaminofluorene	100	72	776	520	10.8^m	7.2^m
	10	66	308	34^b	4.7^m	0.5^b
2-anthramine	10	61	1955	1078	32.0^m	17.6^m
	1	72	1192	485	16.6^m	6.7^m
2-nitrofluorene	100	1727	1931	1911	26.8^m	27.3^m
Solvent Control (DMSO)	-	73	74	70	1.0	1.0
S-9 Control	-	71	68	70	1.0	1.0

---

[a] Strain Tested: TA98
Type of Preparation: Pour Plate
Replications: 3

[b] Experiment error

[m] Indicates positive mutagenic response

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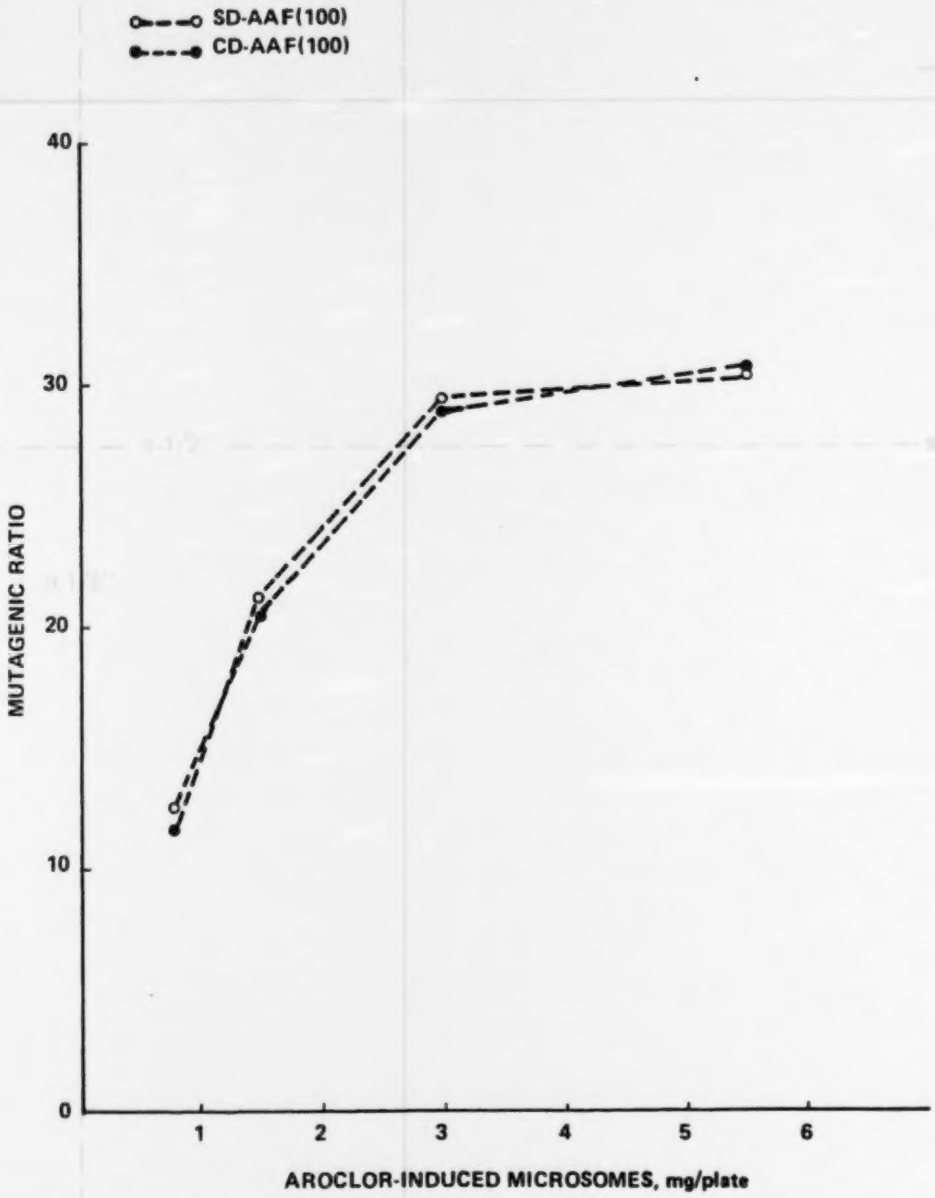

Figure B1. Comparison of Craig-Dawley and Sprague-Dawley Aroclor-induced S9 preparation using TA98 and 2-acetylaminofluorene at 100 μg/plate. (Spontaneous background was 42 colonies.)

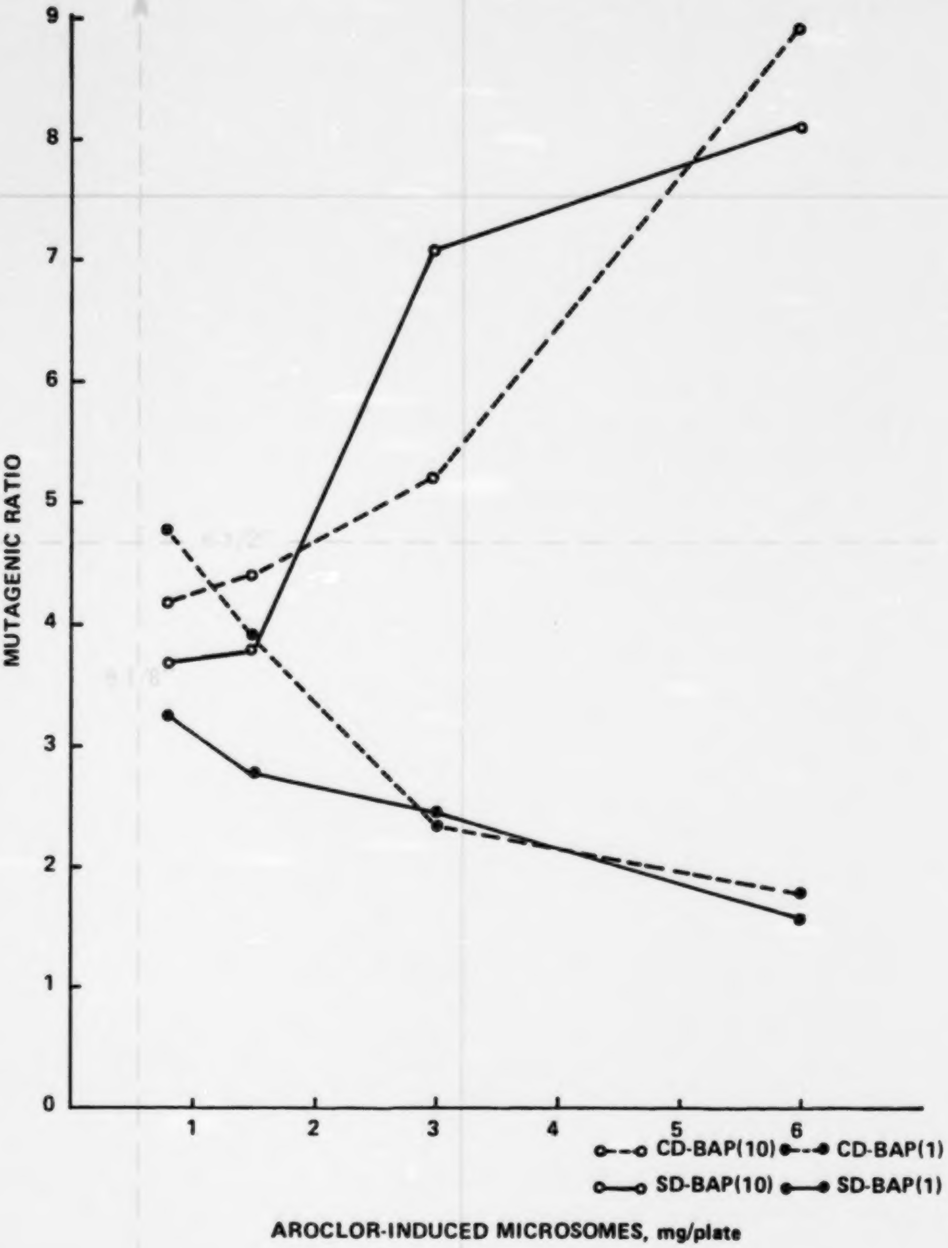

Figure B2. Comparison of Craig-Dawley (CD) and Sprague-Dawley (SD) Aroclor-induced S9 preparation using TA98 and benzo(a)pyrene (BAP) at 10 and 1 μg/plate. (Spontaneous background was 40 colonies.)

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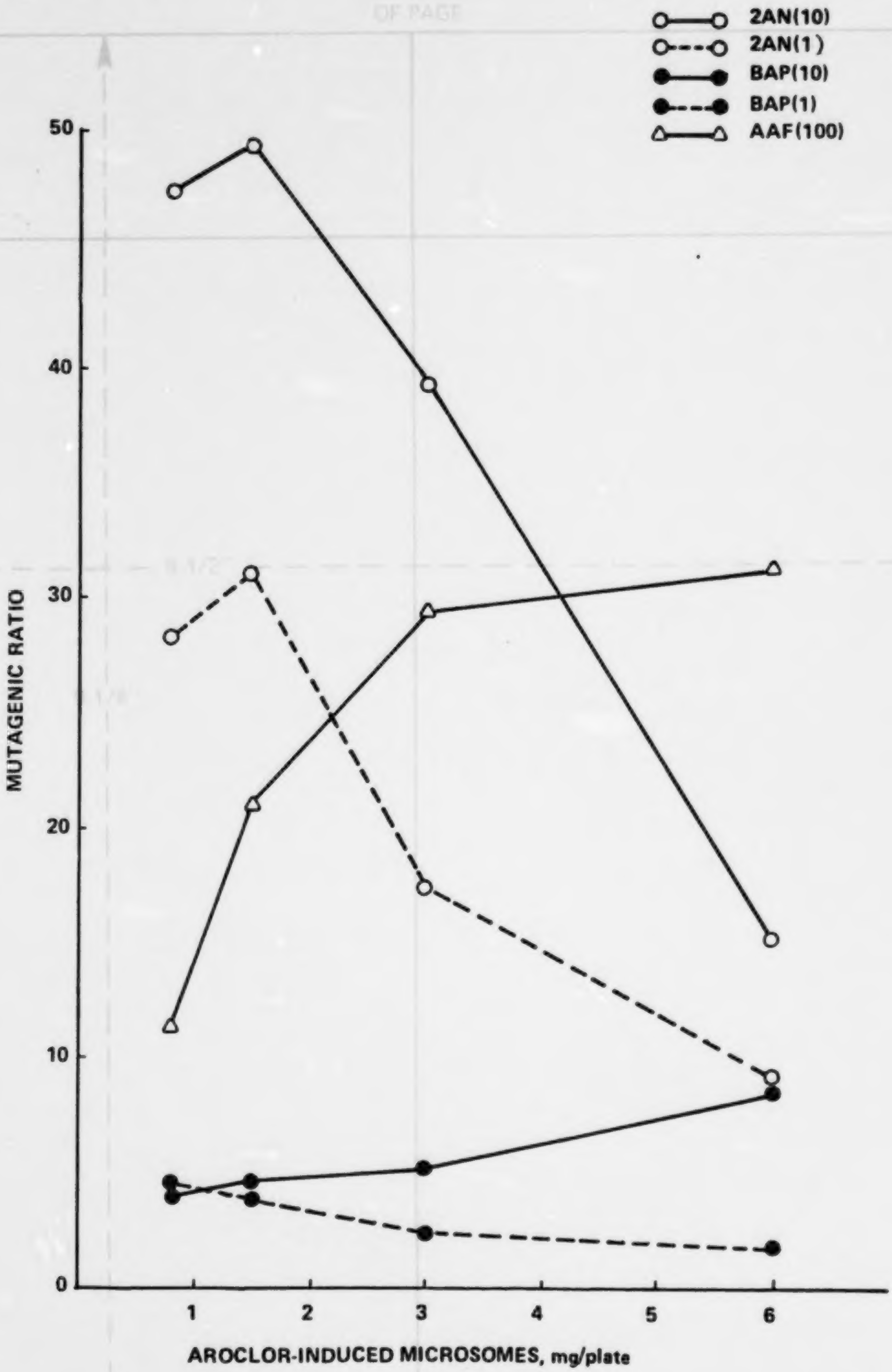

Figure B3. Dose response of Craig-Dawley Aroclor-induced S9 with TA98. (Spontaneous background was 43 colonies.)

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to 200 mg/ml with 0.25 M sucrose. The liver was homogenized (on ice) with four strikes of a cold Potter-Elvenhjem apparatus with a Teflon® pestle. The homogenate was centrifuged for 10 minutes at 9,000 x g. The lipoprotein layer was aspirated off and the protein concentration of the liver supernatant was adjusted to a 30 mg protein/ml with 0.25 M sucrose stock solution after a Lowry protein assay. The stock solution was tested for sterility, quick-frozen in small aliquots (2-5 ml), and stored for a maximum of two months at -80°C (Revco® Ultracold Freezer). For the assay, an aliquot of stock solution was slow-thawed and adjusted to an appropriate concentration of protein in 0.25 M sucrose. NADPH was added at 0.32 mg/plate. Each batch was tested before use with known positive controls [Benzo(a)pyrene (BaP) and 7,12-Dimethylbenzanthracene (DMBA)] using the plate incorporation assay to insure proper mutagenic potential.

Preparation of Base Layer Agar Plates--All tests employed agar plates containing a base layer of agar, prepared as follows:

Mix Bacto-Agar (Difco), 16 g; dextrose, 20 g; and deionized water, 1 liter. Autoclave. Immediately after autoclaving add 20 ml of Vogel-Bonner Medium E solution made up from $MgSO_4 \cdot 7H_2$), 10 g; citric acid $1 \cdot H_2O$, 100 g; K_2HPO_4, 500 g, $NaNH_4HPO_4 \cdot 4H_2O$, 175 g, deionized water, 670 ml, and sterilized by autoclaving.

Maintain agar at 45°C for one hr. Pour 25 ml aliquots into plastic petri dishes (100 x 15 mm) using an automatic plate pourer (Petrimat). Allow to solidify. Incubate overnight at 37°C to check for contamination. Store under refrigeration.

Preparation of Agar Overlays

Overlays and media were prepared, except for minor modifications, according to the method of Ames et al. (1975). Histidine-negative agar overlays were used in mutagenicity tests with positive mutagen controls and with test samples; histidine-positive agar overlays were used with toxicity and sterility tests. Each tube contained 2.5 ml of agar.

Histidine-negative agar for overlays was prepared from NaCl, 5 g; Bacto-Agar, 6 g; deionized H_2O, 1 liter. Immediately after autoclaving, 100 ml of autoclave-sterilized histidine-biotin solution (prepared from D-biotin, 122.2 mg; L-Histidine-HCL·Monohydrate, 76.6 mg; and deionized water, 1 liter) was added. For histidine-positive overlays, preparation was identical to histidine negative overlays except than 39 mg of L-Histidine-HCl·Monohydrate was added to the agar prior to autoclaving.

Preparation of Bacteria

1. Materials

a. Nutrient Broth: Oxoid Broth, 8 g; NaCl, 5 g; and deionized water, 100 ml.

b. Saline Solution: NaCl, 8.5 g; deionized water, 1000 ml.

2. Procedure

a. Stock Cultures

(1) Grow up cultures in nutrient broth.
(2) Add DMSO (8.75% v/v).
(3) Place aliquots in 0.5 dram vials.
(4) Freeze rapidly in ice/acetone.
(5) Store at -80°C.

b. Test Organisms

(1) Using a stock culture, inoculate two 250 ml Erlenmeyer flasks each containing 50 ml nutrient broth, grow for 15 hr in a shaking 37°C water bath.
(2) For agar well diffusion assay, centrifuge cells in 50 ml tubes for 20 min at 2000 rpm at 4°C.
(3) Decant supernatant and resuspend cells in each tube in 50 ml cold saline.
(4) Re-centrifuge (as in 3), decant, resuspend all cells in cold saline.
(5) For plate incorporation assay, use overnight oxoid broth culture (without centrifuging cells).
(6) Prepare two concentrations of bacteria, one for toxicity test and the other for mutagenicity tests using previously established standard curve relating cell concentration and absorbance at 420 nm.

Preparation of Solutions

1. NADPH

Deionized H_2O	1.0 ml
Sigma NADPH	3.2 mg

Dissolve the NADPH by adding cold sterile H_2O in a large sterile test tube. Keep cold.

2. 0.25 M Sucrose

Sucrose	85.6 g
Deionized H_2O	Bring up to 1000 ml, autoclave, keep cold.

Mutagenesis Testing, Plate-Incorporation Method

With S9--To a tube containing 2.5 ml of histidine-negative overlay agar, add 0.1 ml of S9 microsomal preparation, 0.1 ml of NADPH (prepared by dissolving 3.2 mg of Sigma NADPH in 1.0 ml of cold sterile deionized water), 0.1 ml of a solution of test material or positive control compound in dimethylsulfoxide, and 0.1 ml of bacterial suspension (late log overnight culture to give approximately 10^9 cells per plate).

Without S9--Prepare as above, substituting 0.1 ml of 0.25 M sucrose solution for S9 microsomal preparation and 0.1 ml of deionized water for the NADPH solution.

Quality Control

Positive Mutagen Control Testing--Using histidine-negative overlayers, $\sim 10^9$ cells were plated into each dish. Known mutagens are tested to assure that: (1) the strains are active and (2) the S9 preparation was activating promutagens to the desired levels. If known positive controls did not demonstrate proper mutagenic activity, the test was rejected. Control compounds were:

Strain	Without S9	With S9
TA1535	Sodium Azide 200, 20, 2 µg/plate	2-Anthramine 100, 10, 1 µg/plate
TA1537	Quinacrine HCl 250, 100, 50 µg/plate	2-Anthramine 100, 10, 1 µg/plate
TA1538 & TA98	2-Nitrofluorene 100, 10, 1 µg/plate	2-Anthramine 100, 10, 1 µg/plate
TA100	Sodium Azide 200, 20, 2 µg/plate	2-Anthramine 100, 10, 1 µg/plate

Sterility Testing

Sterility tests were conducted with histidine-positive overlay plates, with the amounts of each test component employed in the tests. Components tested were the test sample(s), positive controls, solvent(s), water, 0.25 M sucrose solution, saline, S9 preparation, and NADPH solution.

MUTAGENESIS TESTING, SPOT TEST METHOD

The spot test method was identical to the plate incorporation method (above), with the exception that, rather than being incorporated into the agar overlay, the text substance or positive control was placed on a filter disc (Whatman No. 1 filter paper) on the surface of the overlay (with and without S9 addition). For a test volume of 100 µl, a filter disk of 5 mm in diameter was used. A positive result in the spot test was indicated by a concentric ring of revertant colonies around the filter disk after a two day incubation at 37°C.

DATA HANDLING AND INTERPRETATION

Toxicity Testing

In a histidine-positive overlay, 800-1,000 cells per disk were plated. Tests were performed with and without induced S9. When plates did not contain S9, deionized water replaced NADPH solution and 0.25 M sucrose solution replaced the S9 microsomal preparation. Test compound was added at 0.1 ml/plate in all tests. A viability ratio was calculated as:

$$\frac{\text{no. colonies with sample}}{\text{no. colonies without sample}} = \text{VIABILITY RATIO}$$

A viability ratio below one was an indication of a toxic effect of the sample compound; any number above one was an indication that the sample was nontoxic to the assay system (bacteria and S9).

Mutagenicity Testing

The raw data was obtained as revertants/plate. Standard deviations were calculated for all sample concentrations, and for positive and negative controls in the pour plate assay. Mutagenic ratios were calculate for all sample and control concentrations were the viability (as determined by cloning efficiency) exceeded 50%. Mutagenic ratios of 2.0 or greater, determined on the linear portion of the dose response curve were considered positive. All results were tabulated on a standard printed format. The Ames test data was further subjected to several internal evaluations for validity.

1. Ames published normal ranges for spontaneous reversion rates for each tester strain and for mutation frequencies with specific known mutagens (positive controls) in each of his tester strains. Reversion rates for each strain in each experiment should fall within these ranges.

	DMSO - S9
TA1535	20-50
TA1537	5-25
TA1538	15-40
TA98	20-60
TA100	80-220

It has been reported (deSerres and Shelby, 1979) that the number of spontaneous revertant colonies will increase during a 48-72 hr incubation period. Some of these air particle samples were incubated for 72 hr to overcome toxicity of the samples, since toxicity can delay the appearance of revertants. Slightly increased spontaneous reversion rates were therefore present in these samples with some strains.

2. Variance at each dosage level should not be excessive (>20%). The variance (s^2) and mean ($\bar{X}$) of the plate count (numbers of revertants/plate) were calculated for each dosage level. These same specification apply for DMSO blanks and for positive controls.

3. Plate counts were considered valid only at dosage levels where toxicity was less than 50%.

4. The mutagenic ratio was defined as the average of plate counts at a given dosage divided by the average of plate counts for the appropriate DMSO (blank) control. A sample was defined as showing a mutagenic response in the Ames test when the mutagenic ratio was greater than 2.0 and when three successive doses produced three increasing mutagenic ratio values.

5. For a test to be accepted, all test components must be sterile.

Quality Control and Assurance Procedures; Mutagenesis

Sample Receipt and Dilution Procedures--Upon receipt of samples from the Chemistry Division, samples were immediately stored at 4°C. Dilution with spectral-grade DMSO (N_2 bubbled) was performed under a biohazard hood (yellow lights were used to avoid photodeactivation). Dilutions were placed into sterile scintillation vials and were serially diluted with sterile pipettes; normal sample concentrations were 5 mg to 0.001 mg/ml, depending on solubility and sample amount. Aluminum foil was wrapped around each vial to protect the sample from photodeactivation.

DMSO was chosen as the solvent for all air particulate samples for the following reasons: (1) DMSO is the organic-solvent-of-choice if compounds are not soluble in H_2O (Maron et al., 1981); (2) Since these samples were water-insoluble hydrocarbons, it was decided that all chemicals (both sample and positive controls) should be in the same solvent. DMSO was therefore chosen since the positive control (2-anthramine) was not soluble in H_2O.

Standard Protocol - Ames et al. (1975)--All test components were added at 100 μl/plate, and all test components (except DMSO) were kept at 0°C. All dose levels were performed in triplicate, and duplicate experiments are performed on separate days if sample was available. Both mutagenicity and toxicity were determined using this protocol. For toxicity determination 800-1,000 cells were placed on histidine containing media; for mutagenicity, 10^8 cells were placed on histidine-deficient plates. Both cell populations were obtained from the same nutrient broth culture.

For quality assurance, the test was divided into four parts:

1. Toxicity test
2. Mutagenicity test
3. Positive Control test
4. Sterility test

No assay was considered valid unless all four sections of the assay were performed, and the spontaneous reversion rate for each tester was determined. The assay was discarded if the spontaneous background reversion rate exceeded normally observed values, if positive control values were not acceptable, or if the sterility test indicated contamination of test components.

Salmonella Strain Validation

The following tests were performed to insure that the strains retained the mutations for proper functioning:

1. Growth on histidine-deficient media to insure proper spontaneous reversion rates for each strain.
2. Growth on histidine positive media to insure histidine dependence.
3. Irradiate with UV for 6 sec (TA1535, TA1537, TA1538) or 8 sec (TA98, TA100) to validate UV - sensitivity (retention of ΔuvrB mutation).
4. Validate zone of inhibition generated by crystal violet-impregnated disk to insure that the lipopolysacchloride coat mutation was present (retention of *rfa* mutation).
5. Test Ampicillin resistance for TA98 and TA100 to insure retention of pkM101 R plasmid.

Quality Assurance for Proper Metabolic Activation for S9

After each S9 batch was prepared, the metabolic activation was determined as follows:

S9/plate	Salmonella assay performed with
0.8 mg	DMBA (100, 50, 25, 10, 1 mg/plate)
1.5 mg	
3.0 mg	BAP (100, 50, 25, 10, 1 mg/palte)
6.0 mg	

Each compound was tested at these 4-S9 concentrations with *Salmonella* strain TA98. Each dose level was performed in triplicate. This experiment provided historical comparison data for metabolic activation since S9 preparations vary in activity. A mutagenic ratio of 2.0 on a linear-dose response curve must be produced for each compound or the S9 preparation was discarded. In general, our quality assurance and control requirement are in agreement with those suggested by deSerres and Shelby (1979). Aryl hydrocarbon hydroxylase (AHH) and B(a)P-monooxidase determinations for the S9 preparations were performed by an outside agency (EPA).

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APPENDIX C

DEVELOPMENT OF AN AGAR WELL TEST FOR EVALUATING THE MUTAGENICITY OF AMBIENT AIR PARTICLES

SUMMARY

An agar diffusion well technique was developed for mutagenic screening of chemically fractionated air particulate samples. In this test, the sample(s) and S9 microsomal preparation were placed in wells cut in the agar; soft agar was then added to fill the well, and after solidification, the bacterial overlay was poured over the entire surface. Results were interpreted as follows: (1) a direct-acting mutagen was observed as a ring of colonies around the sample well; (2) a promutagen was observed as a line of colonies between the sample and S9 wells; and (3) toxicity resulted in a clear zone around the sample well.

Based on the *Salmonella typhimurium*/mammalian microsome mutagenicity test developed by Ames and his colleagues, this qualitative test was verified with known mutagens and its sensitivity was compared to that of the spot test. Optimum size, number, configuration, and spacing of wells. In addition to the amount of S9 rat liver microsomal preparation required per well, were determined. A well configuration suited to general screening is presented.

The major impetus for development of this test procedure was the extremely small sample size available for mutagenic testing of air particulate fractions. Advantages of this test included the ability to: (1) measure multiple parameters (dosage, activation requirements, toxicity, and mutagenicity) on a single plate, thereby greatly extending the amount of information obtainable from the available sample; (2) determine positive, negative, and solvent controls on the same plate as the test compound; (3) conduct dose response and multiple compound testing on a single plate; (4) eliminate adsorption of test compound by the filter disc and; (5) eliminate run-off of liquids onto the agar. The utilization of this agar well test, in conjunction with chemical analysis, has greatly aided in the development of a minimal biological and chemical testing protocol.

INTRODUCTION

The Ames' assay (1, 6), based on reverse mutation of histidine-requiring mutants of *Salmonella typhimurium* to the wild type upon addition of a mutagen, is a rapid and reliable screening method for mutagen detection (3, 5, 6, 8, 16, 18). Spot or plate incorporation tests are commonly used (4, 6, 12), with activation requirements for promutagens supplied by addition of an Aroclor 1254-induced mammalian rat liver S9 microsomal preparation (2, 6). The accuracy of this test system (17), along with its low cost, time requirements (2 days), and ability to test a wide variety of compounds (7, 16) has led to wide acceptance of this test for screening.

This assay has been used to test a wide variety of environmental samples and pure compounds (4, 7, 8, 12, 14, 16, 17). In spite of this, little information exists on the potential mutagenicity of such complex mixtures as ambient air. The Salmonella assay has been used by Tokiwa et al. (24) in Japan, Pitts et al. (22) and Flessel (10) in Southern California, and Talcott and Wei (23) in Buffalo, NY, and California to test for potential mutagenicity of ambient air. Chemical extraction of the curde particulate sample was usually done with such solvents as methanol (24), chloroform and acetone (23), methanol-benzene-dichloromethane (22), or acetone and benzene (10). Mutagenic activity was found in all of these cases, usually in samples obtained after a collection time of 48 hr with a high volume sampler. Both ultimate (direct-acting) (10, 22, 23) and promutagens (indirect-acting) (23, 24) were detected in these air particulate extracts.

In the research reported herein, the Ames system was used to screen for potential mutagenicity of ambient air in U. S. cities with high air pollution indices. Mutagenic activity was evaluated with the agar well technique after initial fractionation of the crude particulate sample into five chemical-classes [i.e., acids, bases, polar and nonpolar neutrals, and polynuclear aromatics (PNAs)]. Mutagenically active fractions were further characterized via a gas chromatograph/mass spectrometer/computer (GC/MS/Computer) system.

Particles collected during this air pollution study consisted largely of inert material (~90%), leaving only a small amount of organic extractable material for fractionation, chemical identification, and mutagenicity testing. Therefore, in order to maximize the amount of information obtained from a small sample size, an agar diffusion well test was developed which was capable of determining toxicity, mutagenicity, and activation requirements on a single plate. This method was tested with positive controls and compared with the spot test and plate incorporation procedures (Hughes et al., 1980).

Materials and Methods

Chemicals

NADPH (tetrasodium salt, Type 1) and known positive mutagens (highest purity available) were obtained from Sigma Chemical Company. Dimethylsulfoxide (spectrophotometric grade) and sucrose were obtained from the Fisher Chemical Company. Agar (Bacto-Agar®) was obtained from Difco Laboratories.

Bacterial Strains

S. typhimurium strains TA98, TA1537, and TA1538 (used to detect frameshift mutations) and TA1535 and TA100 (used to detect base substitution) were obtained directly from Dr. Bruce N. Ames (Biochemistry Dept., University of California, Berkeley).

EXPERIMENTAL PROCEDURES

Bioassay System

In general, the procedures for handling the strains and preparing media components were those of Ames et al. (6), with the following exceptions: (1) Craig-Dawley male rat livers were used as the source for metabolic activation (S9) [activation potentials were very similar to Sprague-Dawley male rats (data not shown)]; (2) NADPH was added directly to the plate (per plate, 0.15 ml containing 480 µg NADPH) in place of an NADPH-generating system (11); (3) use of a 2.5 ml agar overlay rather than a 2.0 ml overlay; (4) S9 microsomal preparation was diluted in 0.25 M sucrose at a concentration of 20 mg protein/ml and added at 3.0 mg protein/ test well. Data from our laboratory (not shown) indicated that S9 microsomal concentration in the well test was most effective from 3.0-6.0 mg of protein/ well. The S9 microsomal preparation was obtained from rat injected with Aroclor 1254 (6). Protein concentration of the S9 microsomal preparation was measured by the Lowry procedure (13).

Particulate samples were tested both with and without an S9 activation system. On plates without S9, H_2O replaced the NADPH and 0.25 M sucrose replaced the S9. Bacteria and sample were added in both cases, and each component was added at 100 µl/plate. In the plate incorporation assay, the sample and other components were added into a soft agar overlay; in the spot test, the sample was placed on a 5 mm paper disc (Whatman #1 filter paper), and this disc was placed on top of an agar overlay containing the other components.

Toxicity testing was performed exactly as the plate incorporation assay except that the overlay contained histidine (80 µg) and only 200-300 cells (from the same population as those for mutagenicity testing) were plated per dish.

Base layers of agar were prepared according to Ames et al. (1975), and dispensed in 25 ml aliquots in to 100 x 15 mm plastic petri dishes. After solidification wells were cut with an autoclaved brass cork borer (18 mm i.d.). Prior to cutting wells in each plate, the borer was dipped in alcohol and flame-sterilized. Agar plugs were removed with a sterile spatula. The configuration of wells depended on parameters to be tested. An autoclavable template was made of stainless steel (0.4 mm wall thickness) to ensure uniform spacing of wells and consistency of results. The basic agar well configuration utilized in the mutagenicity testing of solvent extracts from ambient air particulate is shown in Figure C1. Wells 1 and 2 should be placed as high in the dish as possible,

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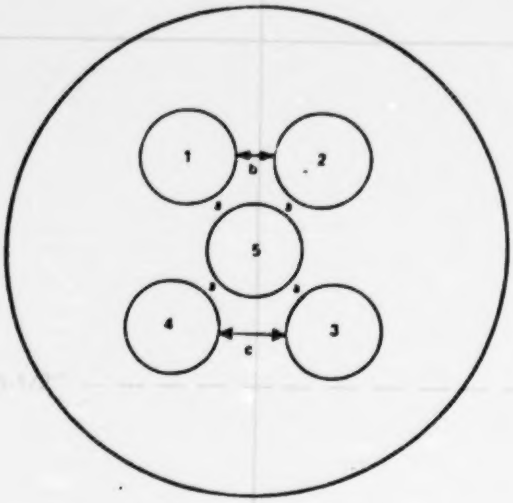

Figure C1. The basic well configuration for mutagenicity testing of ambient air particles in the Ames/Salmonella assay. Wells contained: (1) promutagen positive control (100 µg of 2-anthramine in 100 µl solvent); (2) DMSO, solvent control, (100 µl); (3) low concentration of sample (100 µg sample in 20 µl DMSO); (4) high concentration of sample, (500 µg sample in 100 µl DMSO); (5) Aroclor-induced S9 microsomal preparation [(3.0 mg protein) + 480 µg NADPH in 300 1]. Distances are: (a) 2.5 mm; (b) 5.0 mm; (c) 10.0 mm. The wells are 18.0 mm in diameter.

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to allow the test sample (wells 3 and 4) maximum room to migrate. This asymmetric configuration provided obvious orientation of the wells and insured adequate space for identification of positive results.

After the agar wells were cut in the base agar layer, agar (0.25% in H_2O) was autoclaved, cooled to 45°C in a water bath, and dispensed into all wells in an amount sufficient to give a total well volume of 800 µl. The following components were added to the respective wells: test compound (wells 3 and 4) or positive control (well 1), 100 µl of sample in DMSO/well; S9 microsomal-NADPH preparation (4°C) at 3.0 to 6.0 mg of protein (well 5), 300 µl/well; solvent control (DMSO) (well 2), 100 µl/well. Well components were mixed thoroughly with an automatic pipet (Biopette®, Schwartz-Mann Division of Becton, Dickinson and Co.). The well contents were allowed to solidify (20-30 min). An agar overlay (2.5 ml of agar containing 0.1 ml of an overnight nutrient broth culture which had been washed and resuspended in normal saline to 10^{10} bacteria/ml) was carefully poured over the entire plate. Even distribution of the overlay was essential. Plates were incubated without inversion for 72 hr at 37°C in the dark.

The well test was dependent on relative placement of test compound, S9 microsomal preparation, and solvent control components. Consequently, it was essential that the materials in the wells did not spill out onto the surface of the surrounding agar, and that the agar permited diffusion of the materials into the area immediately adjacent to the wells. An agar concentration of 0.25% was satisfactory for such diffusion. Increasing well agar concentration inhibited diffusion and is therefore not recommended.

The concentration of microsomal preparation utilized in the pour and spot test was 3.0 mg protein per plate. This amount compensated for variation in activation potential from batch-to-batch. In the well test, the amount of microsomal preparation was most effective at 6.0 mg protein per plate. Positive mutagenic response with 2-anthramine increased with increasing amount of microsomal preparation (from 0.8-6.0 mg protein) (Table C1) . However, 2-acetylaminofluorene required a minimum of 6.0 mg protein to show a positive result at the lower concentration of sample (10 and 1 µg). It was therefore decided that 6.0 mg of S9 protein would be employed for routine screening with the well test.

RESULTS AND DISCUSSION

Interpretation of Agar Well Diffusion Test

Qualitative indications of toxicity, mutagenicity and activation requirements can be detected on a single plate with the well test. A positive result in the well test was largely visual and was indicated by colonies appearing in the immediate area of the sample well, either as a curved line of colonies between the sample and S9 wells (indicating a S9 activation requirement [promutagen]) or as a circle of colonies around the entire sample well (indicating a direct acting mutagen). Toxicity was indicated by a clear zone around the sample well. As in the case of

TABLE C1. OPTICAL CONCENTRATION OF S9 MICROSOMAL PREPARATION IN WELL TEST WITH TA1538 (AVERAGE OF TWO PLATES)[a]

Well #	1	2	3	4	5
	Concentration in Well				
Compound	100 μg	Control DMSO	10 μg	1 μg	mg of protein of S9 + NADPH
2-anthramine	18	4	23	27	0.8
	36	1	33	18	1.5
	45	5	30	15	3.0
	22	0	42	40	6.0
2-acetylamino-fluorene	8	1	6	0	0.8
	5	0	0	0	1.5
	28	1	9	2	3.0
	42	3	16	7	6.0

[a] A configuration of wells was as in Figure C1. Compounds were diluted in DMSO and added to their respective wells at 100 μl per well. Numbers are colony counts within a 30 mm diameter circle around each well.

spot tests, problems with interpretation were encountered with compounds that did not diffuse into the agar [*i.e.*, benzo(a)pyrene], or which were extremely soluble and diffused throughout the entire plate (sodium azide). A weak mutagen or one that did not diffuse readily into the agar produced diffuse colonies within the sample well and was detected by total number of colonies in the well itself. Typical background counts in the well test are shown in Table C2. A positive mutagenic response in the well test was qulitative and therefore visual. The background counts are presented here for information purposes only.

Interpretation of positive results with the agar well test was enhanced by using no more than 5 wells, 18 mm i.d., in a standard 100 x 15 mm plate, with a distance of 2.5 mm between the wells containing the test compound and the S9 preparation. The critical factor was the distance the microsomal components migrate, as this was limited to 3-5 mm. In addition, an Aroclor-induced microsomal preparation must be used, since no activity was detected in the well test when a noninduced microsomal S9 preparation was tested.

Verification of Agar Well Diffusion Test

The well diffusion technique was verified with known mutagens including sodium azide, 2-naphthylamine, 2-anthramine, benzo(a)pyrene, 2-nitrofluorene, 2-acetylaminofluorene, quinacrine hydrochloride, and 9-aminoacridine (Table C3). The well test detected all mutagens detectable by the spot test. Benzo(a)pyrene was not detected in either the spot or agar well test because nonaqueous-soluble compounds (such as PNAs) do not migrate in aqueous agar.

Although the agar well test and spot test are qualitative assay techniques, the numerical results for each compound with each test are given for comparison purposes only. The assay-of-choice when adequate sample was available was the standard Ames/*Salmonella* pour plate technique. The absolute numbers in the agar well technique were actually lower than that in the spot test, but it should be realized that the background count in the agar well test were also significantly lower than that in the spot test. For example, background counts for strains TA1535, TA1537, and TA1538 were 2 (avg) for the agar well test and 25 (avg) for the spot test; background count for TA98 and TA100 were 7 (avg) for the agar well test and 75 (avg) for the spot test. Therefore, in comparing the qualitative well and spot test results, the number of colonies produced in the well test become much more significant when the area counted in the well test (30 mm circle around the test well), the low background counts, and the distinctive colony distribution were all taken account.

SUMMARY

An agar well diffusion test, similar in principle to the Ouchterlony immunodiffusion well procedure (1978), was developed to overcome the difficulties associated with minute sample size, and was verified with known mutagens. This agar well test was initially developed to optimize

TABLE C2. TYPICAL BACKGROUND IN THE WELL TEST[a]

	Number of Colonies Appearing in 30 mm Diameter Circle Around Sample Wall	Standard Deviation
TA 1535	3.4	± 1.9
TA 1537	1.5	± 1.2
TA 1538	2.4	± 1.6
TA 98	4.0	± 2.1
TA 100	9.6	± 3.2

[a] Counts were from plates with only NADPH + S9 microsomal preparation (well #5) and DMSO (wells #1, 2, 3, and 4) in wells. 80 wells were counted for each strain. Configuration of wells and amounts of materials were the same as in Figure C1.

TABLE C3. COMPARISON OF RESPONSE OF POSITIVE MUTAGENIC COMPOUNDS IN POUR, SPOT (WITH AND WITHOUT FILTER DISC) AND WELL TESTS

Compound and Strain	Concentration (ug/plate)	Number Colonies Produced (Minus Spontaneous Revertants)			
			Spot Test		
		Pour Plate	(Without Filter Disc)	(With Filter Disc)	Well
TA 1535	100	312 (+)[b]	360 (+)	333 (+)	15 (+)
2-anthramine[a]	10	342 (+)	436 (+)	377 (+)	28 (+)
2-naphthylamine	10	254 (+)	145 (+)	133 (+)	18 (+)
sodium azide	20	1,306 (+)	1,258 (+)	1,285 (+)	1,258 (+)
	2	316 (+)	341 (+)	367 (+)	385 (+)
Background	0	26	24	27	3
TA 1537					
2-anthramine[a]	100	211 (+)	57 (+)	138 (+)	6 (+)
quinacrine hydrochloride	250	429 (+)	427 (+)	TNTC (+)[c]	TNTC (+)
9-aminoacridine	200	1,767 (+)	408 (+)	TNTC (+)	TNTC (+)
Background	0	25	25	27	1
TA 1538					
2-anthramine[a]	100	1,774 (+)	807 (+)	1,069 (+)	43 (+)
	10	1,910 (+)	1,050 (+)	886 (+)	65 (+)
2-acetylaminofluorene[b]	100	797 (+)	249 (+)	233 (+)	18 (+)
benzo(a)pyrene[a]	100	157 (+)	54 (+)	49 (+)	1 (-)
2-nitrofluoride	100	1,599 (+)	549 (+)	418 (+)	307 (+)
Background	0	22	27	25	2
TA 98					
2-anthramine[a]	10	1,584 (+)	674 (+)	1,033 (+)	45 (+)
2-acetylaminofluorene[a]	100	427 (+)	104 (+)	233 (+)	30 (+)
benzo(a)pyrene[a]	100	199 (+)	68 (-)	108 (+/-)	1 (-)
	10	245 (+)	63 (-)	78 (-)	1 (-)
2-nitrofluorene	100	2,496 (+)	648 (+)	600 (+)	391 (+)
Background	0	75 (+)	77	72	4
TA 100					
2-naphthylamine[a]	10	210 (+)	85 (+)	92 (+)	20 (+)
2-anthramine[a]	100	368 (+)	236 (+)	248 (+)	23 (+)
	10	559 (+)	155 (+)	233 (+)	21 (+)
sodium azide	2	266 (+)	187 (+)	243 (+)	126 (+)
Background	0	80	75	77	10

[a] Aroclor-induced S-9 microsomal preparation needed

[b] + = mutagenic, - = non-mutagenic, +/- = weakly positive

[c] TNTC = too numerous to count

testing capabilities with a limited amount of sample, but it could also allow a number of other parameters to be measured. Co-mutagens, synergistic/ antagonistic effects, different solvents or inducers, or different microsomal preparations can easily be compared with the agar well test. Numerous configurations are possible and can be adjusted to suit specific needs.

The well test was effective in detecting different chemical-classes of mutagens. The major disadvantage of this is qualitative test was that this test relied on the ability of the sample to migrate in the agar, as does the spot test. Consequently, PNAs or similar hydrophobic mutagenic compounds may not be detected with these tests. In addition, spot and well tests are only qualitative indicators. For reliable quantitative data when sample size is not limited, the plate incorporation test, which produces a reproducible dose-related mutagenic response, is the method-of-choice.

A priority scheme was adopted for the testing of air particulate samples with the agar well and pour plate techniques, in order to maximize the amount of information obtainable (Table C4). An agar well test was utilized on all chemical-classes that contained less than 10 mg, except for the PNA fraction, where a plate incorporation test with an Aroclor-induced S9 microsomal preparation and strain TA98 was utilized. If enough sample remained, plate incorporation tests were performed with S9 and then with no S9. A dose response curve was obtained whenever possible. Strain utilization for the well test, in order of decreasing priority was; TA98, TA1535, TA1537, TA1538, and TA100. TA100 was placed last in this particular priority listing because the high spontaneous revertant frequency of this strain (80-220) interferred with the interpretation of the results.

The development of the agar well diffusion test increased the mutagenic testing capabilities with ambient air particulate samples. This agar well test, when coupled with chemical fractionation and identification, greatly facilitated the rapid evaluation of potential mutagens in ambient air.

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TABLE C4. BIOASSAY PRIORITY SCHEME

(1) Sample amount >10 mg
Plate incorporation method utilized at 5 dose levels. The S9 (Aroclor-induced) concentration was 3.0 mg of protein/plate. Duplication plates for each strain were used in the following order: TA98, TA100, TA1535, TA1537, and TA1538. Toxicity testing was performed at highest dose level only. Preferred initial dose ranges were 1,000, 500, 100, 10 and 1 μg/plate, S9 addition first, then no S9.

(2) Sample amount <10 mg
Agar well diffusion test was utilized at 2 concentrations (*i.e.*, 500 and 100 μg/plate) except for PNA fractions which were tested with pour plate, 500 μg, Aroclor-induced S9 and TA98. Duplicate plates were tested in the following order: TA98, TA1535, TA1537, TA1538, and TA100. Sample was diluted to one concentration for all tests; to obtain lower test concentrations, less sample volume per well was used. Toxicity testing was at 500 μg with TA98 and TA1535 using 1,000 colonies/plate.

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APPENDIX D

ABSTRACTS AND PUBLICATIONS

ENVIRONMENTAL MUTAGEN SOCIETY (EMS)
9th ANNUAL MEETING
March 9-13, 1978
San Francisco, California

APPLICATION OF AGAR DIFFUSION WELLS FOR MICROBIAL MUTAGENESIS TESTING OF AIR POLLUTANTS. T. J. Hughes and L. Little, E. Pellizzari, C. Sparacino, Research Triangle Institute, and L. Claxton and M. Waters, EPA Reserach Triangle Park, NC 27709

Little information exists on potential mutagenicity of complex mixtures such as particulates in air collected in urban areas. A major limitation in application of the Ames *Salmonella typhimurium* reverse mutation detection system to such mixtures is the minute amount of particulate material in relation to volume of air sampled. An agar well system was developed which allows multiple parameters (dose, activation requirements, toxicity, synergistic effects) to be tested on a single plate, substantially decreasing the total amount of material required. The system was validated with known mutagens and five *Salmonella* strains. Optimum size and spacing of wells were determined; various configurations of wells were tested. Results suggest that with this procedure microbial mutagenesis testing can be employed in mass screening for preliminary assessment of mutagenic effects of environmental samples, even in cases where the total amount of crude sample is small. Research was supported by EPA Contract No. 68-02-2724.

APPLICATION OF SHORT-TERM BIOASSAYS IN THE FRACTIONATION AND
ANALYSIS OF COMPLEX ENVIRONMENTAL MIXTURES
Williamsburg Virginia
February 21-23, 1978

MICROBIAL MUTAGENESIS TESTING OF AIR POLLUTION SAMPLES

T. J. Hughes, Research Triangle Institute, Research Triangle Park, NC;
L. Little, L. Claxton, M. Waters, E. Pellizzari, C. Sparacino.

Air samples from U. S. cities with known high air pollution indices were screened for mutagenic activity using Ames' Salmonella typhimurium reverse mutation detection system. Objectives of the study were (1) identification of possible sources of mutagenic pollutants, (2) determination of capability of Ames' test to detect such pullutants, and (3) modification of the test for mass screening purposes. Particulates collected from S. Charleston (WV) with the Battelle Maxi-Sampler were partitioned into fractions which were chemically identified and tested for mutagenic activity with five tester strains (TA98, TA 100, TA 1535, TA 1537, and TA 1538). Compounds were tested for toxic and mutagenic activity with and without metabolic activation. Because of the minute amount of material available in each fraction (0.2-17 mg), spot tests were used initially and fractions showing activity were subsequently subjected to pour plate testing. None of the fractions were toxic under the conditions tested. Preliminary results suggest mutagenic activity in fractions containing organic bases, acids and aromatics. Marginal activity was detected with non polar acids and neutrals. Each of the five tester strains gave a positive response with at least one of the active fractions. Of those fractions showing mutagenic activity, only the aromatic nonpolar acids and neutral fractions required metabolic activiation. Sensitivity of the spot tests was improved by increasing histidine concentration in the overlay. Results of pour plate tests confirmed spot tests; however, activity was somewhat lower in pour plate tests due to sample dilution. Results suggest that the assay can detect mutagenic activity in small amounts of crude mixutres and fractions, but the major problem is still availability of the sample amounts. A system allowing testing of multiple parameters (such as activation requirements and dose response effects) on a single plate, thereby decreasing total sample requirement, is underway. Research was supported by EPA Contract No. 68-02-2724.

APPLICATION OF SHORT-TERM BIOASSAYS IN THE FRACTIONATION AND
ANALYSIS OF COMPLEX ENVIRONMENTAL MIXTURES
Williamsburg, Virginia
February 21-23, 1978

INTEGRATING OF MICROBIOLOGICAL AND CHEMICAL TESTING INTO THE SCREENING OF AIR SAMPLES FOR POTENTIAL MUTAGENICITY.

E. D. Pellizzari and L. W. Little, Research Triangle Institute, Research Triangle Park, NC; C. Sparacino, T. Hughes, L. Claxton, M. Waters.

Compared to whole animal or plant assays, microbial mutagenesis assays required less space, sample and time for completion. Conducted concurrently with chemical testing, they provide a rapid indication of fraction activity, thus denoting components of high priority for further testing. Volatile and aerosol phase organics from ambient air are being collected from urban-industrialized and rural areas in the Continental U. S. and are being assessed by two independent mutagenic bioassays - *Tradescantia* flowering plant and Ames' *Salmonella typhimurium* system. Volatile organics are trapped on a large (22 g) Tenax GC cartridge, then thermally desorbed and transferred to the Ames bioassay. Particulate phase organics are collected (with a Batelle Maxi-Sampler) in three sized: ≥ 3.5 μ, 1.7-3.5 μ, and ≤ 1.7 μ, and extracted with cyclohexane and methanol using sonification. Some 5% (wgt) of the particulate mass is removed as organic extractables. Extracts are fractionated using a validated solvent partition scheme into 5 fractions: acids, bases, non-polar neutrals (paraffins), PNA's and polar neutrals. Fractions are divided into aliquots for bioassay and for qualitative chemical analysis. Separation and identification of fractional components are accomplished by capillary gc-ms. Quantitation is performed on specific compounds of known high hazard potential. In application of the tests to air samples major problems arise since (1) the minute amount of material which can be collected even with the Maxi-Sampler limits the number of chemical and microbial tests which can be conducted; (2) with vapors and gases, containment and quantitative delivery to the system must be assured. The Ames test has been modified to allow testing of multiple parameters in a single plate, thus reducing total amount of material needed. In development is a test system in which gases and vapors sorbed to carbon are desorbed in the plate itself, confining volatiles in a small space and allowing maximum exposure of the organisms. Research was supported by EPA Contract No. 68-02-2724.

ENVIRONMENTAL MUTAGEN SOCIETY
11th ANNUAL MEETING
March 16-19, 1980
Nashville, Tennessee

THE MUTAGENICITY OF FRACTIONATED ORGANICS EXTRACTED FROM AMBIENT AIR.

Alan Kolber, T. Hughes, T. Wolff, Research Triangle Institute, Research Triangle Park, NC 27709. M. Waters, L. Claxton, J. Huisingh, Genetic Toxicology Division, Health Effects Research Laboratory, U.S. EPA, Research Triangle Park, NC 27709.

Ambient air particulate was collected from five geographical locations within the U.S. during 1977 and 1978. The organics were extracted with cyclohexane-methanol and partitioned into six chemical classes, using a chemical fractionation scheme validated with a mixture of know compounds. The fraction of the mass of total organic extract was calculated for each chemical class, as well as total recoveries after partitioning. The composition of each chemical class was examined by GC/MS and each fraction assayed for mutagenicity using a modification of the Ames/*Salmonella* bacterial bioassay. The air was sampled from locations near Houston and Beaumont, TX; Lake Charles, LA; Elizabeth, NJ; and Upland, CA, using a Battelle High Volume (Maxi) Sampler for periods of one week or more. The particulate was collected onto electrostatic precipitator screens designed to separate the material into three size categories (<1.7 μ, 1.7-3.5 μ, and >3.5 μ dia). Mutagenicity assays were quantitative (pour-plate) when sample size allowed and qualitative (agar diffusion well-test) when sample size was limited. Site-related trends for mutagenicity were observed. The polynuclear aromatic hydrocarbon fractions were generally the most mutagenic for each site, although the mutagenicity of other fractions varied from site to site.

Supported by U.S. EPA Contract 68-02-2724.

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APPLICATION OF SHORT-TERM BIOASSAYS IN THE FRACTIONATION AND ANALYSIS OF COMPLEX ENVIRONMENTAL MIXTURES

Williamsburg, Virginia
February, 1980

COLLECTION, CHEMICAL FRACTIONATION, AND MUTAGENICITY BIOASSAY OF AMBIENT AIR PARTICULATE.

A. Kolber, T. Wolff, T. Hughes, E. Pellizzari, and C. Sparacino, Research Triangle Institute, Research Triangle Park, NC 27709. M. Waters, J. Huisingh, and L. Claxton, Health Effects Research Laboratory. U.S. EPA, Research Triangle Park, NC 27709

Introduction

Our industrial society has created thousands of synthetic xenobiotics to support our modern lifestyle. Many of these substances, and their by-products, enter our atmosphere in the form of vapor-phase and particulate pollutants that can be ingested by respiration and skin contact. These results to human health and the ecosystem from these airborne organic pollutants are due mainly to polycyclic organic matter, specific industrial emissions such as halogenated hydrocarbons, and end-use chemicals such as pesticides (Fishbein, 1976). Polycyclic organics associated with air particulates are believed to result primarily from incomplete combustion of organic matter (Moller and Alfheim, 1980). These carcinogenic components, [benzo(a)pyrene [B(a)P], benz(a)anthracene] and other polycyclics have been well characterized chemically (Shubik and Hartwell, 1969). As population and industrial activity increase, the growing health hazards associated with ambient air pollution must be further assessed and evaluated.

Air pollutants are chemically complex environmental mixtures, whose compositions vary geographically with local industrial activity and weather conditions. Seasonal variations in chemical composition and mutagenicity of organic extracts of air particulate have been documented in Oslo, Stockholm, and New York City (Moller and Alfheim, 1980; Lofroth, 1980; Daisey et al., 1979). All three communities exhibited higher mutagenicity during the winter months, and in each case, a major contributing factor was polynuclear aromatic hydrocarbons (PAH). These indirect-acting mutagens, which require metabolic activation, were produced from combustion of heating oil.

Ambient air particulate matter has been estimated at 56 x 10^{13} g/yr, total global load, including natural pollutants such as dusts, agricultural particulate, and others. The man-made (anthropogenic) global load is estimated to be 28 x 10^{13} g/yr. Vapor-phase pollutant organics are believed to represent 60 to 90% of the total organic load; the remainder is organics adsorbed onto particulate matter (Hidy and Brock, 1970; Duce, 1978).

Chemical monitoring and analysis of air pollutants have been conducted for many years, but only within the last decade has biotesting technology improved enough to permit simultaneous chemical and biological characterization. However, chemical-analytical expertise exceeds the present biological capabilities, both qualitatively and quantitatively (Hughes et al., 1980).

In this study, air particulate was collected, size-fractionated, solvent-extracted and fractionated into chemical classes, which were then characterized by GC/MS/Computer analysis. These chemical class fractions were then biotested for mutagenic activity using the Ames/*Salmonella* bacterial mutagenicity assay. Vapor-phase organic pollutants were also collected and tested. However, no quantitative method was available to adequately measure the mutagenicity of the vapor-phase components; consequently, we are presently developing a bioassay protocol capable of quantifying the mutagenicity of vapor-phase substances. It should be noted that mutagenicity or carcinogenicity is by no means the only significant health hazard that could be presented by air pollutants. As additional *in vitro* bioassay capabilities are developed, other potential toxicity parameters will be examined, such as neurotoxicity and lung toxicity.

AMBIENT AIR POLLUTANTS: COLLECTION, CHEMICAL CHARACTERIZATION AND MUTAGENICITY TESTING. T. J. Hughes, E. Pellizzari, L. Little C. Sparacino and A. Kolber, Research Triangle Institute, P. O. Box 12194, Research Triangle Park, NC 27709. Mutation Research *76*, 51-83 (1980)

This review discusses sources and types of air pollution; collection, fractionation and chemical analysis of air samples; and *in vitro* bioassay systems capable of quantitating mutagenicity of these air samples and their components. Case studies utilizing bioassays to detect mutagenicity, such as the Tradescantia plant assay (on-site) and the Ames/Salmonella bacterial assay (off-site) are reviewed. Problems such as sample size, complexity of the air sample (both vapor-phase and particulate), and artifact generation are discussed. The state-of-the-art techniques for air-pollution analysis (an integrated biological and chemical approach) are reviewed, and directions for future research are recommended.

Methods for collection of air particulate and vapors are reviewed, as are solvent extraction and further fractionation of particulate organic matter into various chemical groups (*e.g.*, acid, base, PNA, PN, NPN). Methods for chemical identification of individual components are included.

Experimental results comparing Tradescantia with Ames/Salmonella mutagenicity studies are presented (certain vapor-phase organic substances were highly mutagenic for Tradescantia plant stamen hairs). Bacterial mutagenesis test results of air particulates and their extracts indicated the major portion of the activity resided in the acid, base and PNA fractions. Different type of mutagens identified in fractionated air-particulate extracts are discussed, and recommendations for the use of integrated chemical-analytical and biological screening systems in further studies of toxic air pollutants are proposed.

APPLICATION OF SHORT-TERM BIOASSAYS IN THE ANALYSIS OF COMPLEX ENVIRONMENTAL MIXTURES

January 25-27, 1982
Chapel Hill, North Carolina

DEVELOPMENT OF A MODIFIED PREINCUBATION TECHNIQUE FOR DETECTION OF MUTAGENIC VAPOR-PHASE ORGANIC COMPOUNDS.

T. J. Hughes, T. J. Wolff, A. R. Kolber, C. M. Sparacino, and E. D. Pellizzari, Research Triangle Institute and L. Claxton, J. Lewtas, and M. D. Water, EPA Research Triangle Park, NC 27709

ABSTRACT

Vapor-phase organic compounds (e.g., vinyl chloride) have been identified as weakly mutagenic in the Ames/*Salmonella* assay. This classification of mutagenic potential however may be due to an inappropriate method of detection rather than to an actual weak mutagenic response. The standard Ames/*Salmonella* pour plate technique [Ames, et al., Mutation Res. 31, (1975) 347-364] is not adequate to detect the mutagenicity of organic compounds with high vapor pressures because this technique requires incubation of the sample at 45°C. Substances which boil below this temperature may volatilize before interacting sufficiently with the bacteria, thereby preventing the test system from detecting mutagenic compounds. Although certain methods (such as placement of agar plates in a dessicator) have been utilized to study the mutagenicity of vapor-phase organic compounds in the Ames/*Salmonella* assay, these methods were not quantitative due to the high volatility and aqueous insolubility of vapor-phase mutagens.

Consequently, a quantitative technique to measure the mutagenic potential of vapor-phase organic compounds in the Ames/*Salmonella* assay was developed. The liquid suspension preincubation assay of Yahagi et al. [Cancer Lett. 1 (1975) 91-96] was modified to allow optimal association of bacteria and vapor-phase compounds, thus increasing the sensitivity of vapor-phase mutagen detection. Modifications which increased the sensitivity of detection to known vapor-phase mutagens included: use of logarithmically grown *Salmonella* tester strains rather than stationary grown cells; use of liquid preincubation rather than the standard pour technique; addition of histidine and biotin-containing agar to the liquid suspension, and incubation at 37°C in a closed system. Parameters investigated with this preincubation system included: incubation time and temperature; growth phase of tester strains, stabilization of S9, solvents, toxicity evaluations, and long term chemical and biological stability of vapor-phase compounds at -80°C. The results from the preincubation assay were evaluated against those of the standard pour plate assay with log and stationary phase cells. Known vapor-phase organic mutagens evaluated with this protocol included: butylene oxide, propylene oxide, ethylene oxide, ethylene dibromide, styrene

oxide and vinylidine chloride. Mutational frequencies of these vapor-phase compounds were increased by a factor of 3 or more in the preincubation assay. This increase in sensitivity as well as the lower variability of the preincubation technique was statistically validated. The liquid suspension preincubation assay is suitable for the evaluation of cryogenically frozen ambient air vapor-phase organic field samples. The utilization of this preincubation technique in tandem with GC/MS analysis of Tenax® trapped vapors allows quantitative chemical and biological analysis of potentially mutagenic ambient air vapors. This research was sponsored by EPA Contract No. 68-02-2724.

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ENVIRONMENTAL MUTAGEN SOCIETY (EMS)
13th ANNUAL MEETING
February 26 - March 1, 1982
Boston, Massachusetts

EVALUATION OF A MODIFIED PREINCUBATION TECHNIQUE FOR DETECTION OF MUTAGENIC VAPOR-PHASE ORGANIC COMPOUNDS,

T. J. Hughes[1], T. J. Wolff[1], A. R. Kolber[1], C. M. Sparacino*[1], E. D. Pellizzari*[1], L. Claxton[2], J. Lewtas[2], and M. D. Waters[2], Research Triangle Institute[1], Post Office Box 12194, RTP, NC 27709 and Environmental Protection Agency[2], RTP, NC 27711.

A quantitative technique to measure the mutgenic potential of vapor-phase organic compounds in the Ames/*Salmonella* assay was developed. The standard Ames/*Salmonella* pour plate technique [Ames et al., Mutation Res. 31 (1975) 347-354] was not adequate to detect the mutagenicity of organic compounds with high vapor pressures because this technique required incubation of the sample at 45°C. The liquid suspension preincubation assay of Yahagi et al. [Cancer Lett. 1 (1975) 91-96] was therefore modified to allow optimal association of bacteria and vapor-phase compounds. Modifications to increase the detection of known vapor-phase mutagens included: use of logarithmically grown *Salmonella* tester strains rather than stationary-phase cells, use of liquid preincubation rather than the standard overlay technique, addition of histidine and biotin-containing agar to the liquid suspension, and incubation at 37°C in a closed system. This modified preincubation technique was tested with pure mutagenic vapor-phase organic compounds (ethylene oxide, propylene oxide, butylene oxide, styrene oxide, ethylene dibromide and vinylidene chloride). The preincubation technique was significantly more sensitive (3x or more), and less variable than the standard pour plate technique. Vapor-phase field samples collected on Texas® cartridges contained signature mutagenic/carcinogenic compounds identified by GC/MS/Computer (*e.e.*, vinylidene chloride). This preincubation technique, in tandem with GC/MS analysis of Tenax® trapped vapors, allows quantitative chemical and biological analysis of ambient air vapors. Sponsored by EPA Contract No. 68-02-2724.

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